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RELATIONS IMMUNOLOGIQUES ENTRE VIBRIO CHOLERAE
(FORMES LISSES, RUGUEUSES ET MUTANTS) ET LES
VIBRIONS NON AGGLUTINABLES (NAG)

Immunological interrelationships of smooth,
rough and mutant forms of Vibrio cholerae and
so-called NAG Vibrios

THESE

présentée à la Faculté des sciences de l'Université de Genève pour obtenir
le grade de docteur ès sciences, mention biologique

par

Ghassem Jahangeer SAHAMI
de Téhéran (Iran)

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La Faculté des sciences, sur le préavis du professeur ordinaire R. H. REGAMEY, directeur de thèse, et d'une commission composée du professeur ordinaire G. TURIAN, responsable devant la Faculté des sciences, et du docteur ès sciences J. C. PIFFARETTI (Faculté de médecine), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

GENEVE, le 15 octobre 1974

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Le Doyen :

Jean MULLER

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I. INTRODUCTION

Vibrio cholerae produces a disease which may range from an almost sub-clinical form to mild or severe diarrhea. Asymptomatic carriers are known as well. But cholera, in its severe form, is an acute profound diarrheal disease which could be fatal. The syndrome of the disease is entirely due to the massive loss of isotonic fluid from the small bowel, which leads to severe dehydration and acidosis as a consequence of electrolyte imbalance in the human body. It is only a little more than a decade since it has been shown that pathogenesis of cholera is due to a protein exotoxin, elaborated by the organism in the small bowel. Recent studies with purified cholera exotoxin have contributed a great deal to the present understanding of the mechanism of action of this toxin, and its relationship to the disease.

Most of the present evidence indicates (51, 76) that the effect of the toxin is functional, without any damage to cell structure. The toxin causes the increased flow of chloride ions and as a consequence of chloride movement, the sodium ions flow as well from the plasma to the bowel lumen. The active sodium ions transport (and chloride ions as a consequence of the sodium movement) from lumen to plasma remains unchanged.

The present acceptable mechanism (32, 33, 54, 76) is that when mucosal cells are exposed to the cholera toxin, the toxin combines with certain receptors of ganglioside nature, located on the brush border of the mucosal cell. There is evidence that cholera toxin consists of two components : the larger component, containing four sub-units, combines with the gangliosides and facilitates the entry of the other component, which is responsible for the activity of the toxin in the cell. The toxin stimulates and increases the activity of adenylyl cyclase, an enzyme present in lateral and basal membranes of the cells. Adenosine 3', 5' -cyclic monophosphate (cAMP) is formed from ATP under the influence of adenylyl cyclase. Therefore the consequence is the increased formation of cAMP. It is hypothesized that cAMP, in its turn, combines with the inhibitor portion of a protein kinase, liberating the active portion of the enzyme. The enzyme activates the transfer of the phosphate group from ATP, which phosphorylates unknown protein(s), which causes excess chloride ion transport from plasma across the cell to the intestinal lumen. Sodium ion has to accompany this chloride ion secretion. The concentration of sodium chloride in the lumen increases the osmotic pressure, and the flow of water to the bowel lumen takes place, resulting an isotonic watery diarrhea.

In the past, 6 pandemics are known to have been caused by V. cholerae, which spread from its own endemic foci of West Bengal State, India. The 6th pandemic subsided approximately in the 1940's. Since 1961 (4, 22, 44), a new pandemic started from the island of Sulawesi (Celebes, in Indonesia) and its ende-

mic wave has reached Africa and some European countries; it is caused by cholera vibrio (El Tor), which is believed to have been the endemo-endemic strain before 1961. The development of the seventh pandemic stimulated research on cholera on various aspects, including the pathophysiology of cholera mentioned above. However, many problems are still to be solved, among these the role of mutants of V. cholerae and their importance from the epidemiological point of view.

The existence of a heatstable O somatic, as well as thermolabile H flagellar antigens has been known for a long time. Somatic antigenic variation and serological races of V. cholerae have been studied by several investigators (2, 23, 60, 62, 63, 64, 77). The findings of Gardner and Venkatraman (1935) are the foundation of our present concepts of the antigenic structure of cholera and cholera-like vibrios. By antigenic analysis of numerous strains of cholera and cholera-like vibrios, using cross-agglutination and absorption techniques, these authors found a number of specific but diversified heatstable O antigens, which were the basis for their classification. Each antigen being shared by several strains, it was possible to put the majority of the vibrios on hand in six serological groups, referring to them as subgroups O-I to O-VI. According to this classification, which has been almost universally accepted, all V. cholerae, both classic and El Tor, fall in subgroup O-I; the other vibrios, which for the moment are referred to as non-agglutinable (NAG), belong to subgroups O-II to O-VI. The El Tor classical strains Ogawa, Hikojima and Inaba are subtypes within subgroup O-I. In addition to the main O antigens of subgroup O-I, the Inaba and Ogawa types each have a different auxiliary O component, which cross-reacts with antisera prepared against other subgroups of vibrios. On the basis of the nature of H as well as O antigens, the cholera group consists of vibrios having a specific heatstable O antigen and a common heatlabile H antigen, and similar biochemical characters. These findings have subsequently been confirmed by several investigators (81, 82, 93, 96).

Kabeshima described (1913) two serological types of V. cholerae (Ogawa and Inaba), but in 1923, Nobechi proposed that a third type also exists, possessing the antigenic character of both previously described types. However Gardner and Venkatraman could not definitely confirm the third type (77) ! Burrows et al. (1946) agreed with the characterization and serotypic arrangement previously adopted by Nobechi (1933) and White (1937), but he proposed to change the symbols : an antigenic factor A is specific for the group while factors B and C are type specific (77), so that the three proposed serological types of V. cholerae and their antigenic combinations are as follows :

Inaba (original type)	AC
Hikojima (intermediate)	ABC
Ogawa (variant)	AB

Although the above classification has been accepted by the majority of investigators, Kauffmann (1950) suggested Hikojima and Ogawa strains be considered as one common type, because by cross-absorption tests, they are shown to be identical. In 1964 Felsenfeld demonstrated that the basic biochemical composition of V. cholerae Classic and El Tor is the same, with no significant difference in their RNA, DNA and amino acid compositions.

After the phenomenon of microbial dissociation was explained in Salmo-

nellae and some other organisms, Shousha (1924) gave a full description of the properties of smooth and rough forms of V. cholerae (77). Since then a great amount of work has been accomplished regarding the cultural and immuno-serological relationship of S-R variation and the studies of White (1934, 1935, 1936, 1937, 1938, 1940) have led to a rather full understanding of the variation in this organism.

In order to classify rough forms of various vibrios, White (1935) examined the serological relationship of rough forms of various O subgroups. In addition to naturally isolated rough strains, he studied rough forms obtained by cultivating smooth strains in liquid media with homologous specific serum and complement added. He also isolated certain strains, termed Rho forms, from rough strains cultured in the presence of their homologous R sera and complement. He showed that the rough forms lost the type specific O antigens : they were no longer agglutinated by the antisera of the smooth form. Vibrios belonging to the same smooth antigenic type or subgroup have a common rough antigen, stable in character; for example, the R forms of V. cholerae are serologically identical, irrespective of their serotype. In addition the author stated that the antigenic structure of rough forms derived from smooth forms of different subgroups may somehow be similar and able to be placed in a single large R group.

White (1936, 1937, 1940) described the existence of four distinct polysaccharide groups antigenically responsible for specificity of S, R and Rho forms in V. cholerae. All four are present in the smooth state, but one is dominantly active! After roughening, the smooth active component is lost, while a different component becomes exposed and dominantly active. In the Rho state, only two components are left, with one being dominant.

The term NAG (non-agglutinable) has been adopted for vibrios which are not agglutinable by antiserum against V. cholerae (O subgroup-I). These NAG vibrios are significant from at least two points of view. The first is correlated with its possible role as cholera reservoir in endemic areas : it has been postulated by several investigators (13, 75, 77, 86) that V. cholerae in natural waters mutates to a non-agglutinable form and persistently survives in such a form during the inter-epidemic period; NAG forms would thus play the role of cholera reservoir in endemic areas. Secondly, they may be pathogenic and promote cholera-like diarrhea : there is definite evidence (1, 17, 40, 67, 68, 95) that some limited cholera-like epidemics have been associated with certain strains of NAG vibrios; these NAG vibrios have been able to produce exotoxin (16, 46, 108, 109, 110), which is similar to the exotoxin of V. cholerae.

Following the work of Gardner and Venkatraman, Gallut (1962) examined the O antigenic behavior of nearly fifty strains of NAG vibrios and classified them into 24 O subgroups. In order to clarify the taxonomic position of the so-called NAG vibrios, Sakazaki et al. (1967) have done detailed studies on 164 strains originally considered as NAG vibrios. From these, 22 strains were excluded in preliminary biochemical tests, being either Vibrio parahaemolyticus or other organisms which do not belong to genus Vibrio. The remaining 142 strains were divided into two biotypes according to their physiological and biochemical characteristics. While in basic primary morphological and biochemical tests these two groups were similar; however they differed from each other in several more specific aspects; biochemically the characteristics of the first

group were identical with those of the genus Vibrio, but the biochemical characteristics of the second group were similar to those of Aeromonas formicans. The base composition of DNA was determined in both groups. In the first group, the content of guanine plus cytosine was 44.8%, a value close to that of the Vibrio species; the content of guanine plus cytosine of the second group was 62.0%, a value close to that which has been reported for Aeromonas hydrophila. By numerical analysis also it was shown that these two groups are well distinguishable from each other; the majority of the first group were associated with strains of V. cholerae El Tor, and second group with strains of Aeromonas formicans. Several strains of the first and second groups were inoculated into the gut of the infant rabbits; every strain of the first group produced diarrhea similar to that caused by V. cholerae, but no strain of the second group gave a similar symptom. On the basis of three conventional methods (biochemical characteristics, numerical classification and taxonomy dependent upon the DNA homology test), the authors have suggested that the first group, although non-agglutinable with O antiserum of V. cholerae, should be placed in the same species, V. cholerae.

Finally Sakazaki et al. (1970) by studying the serological properties of a large number of cholera-group vibrios, including cholera and NAG vibrios, established 39 O antigenic groups from which O group I was designated to cholera and the rest to NAG vibrios. Furthermore, they showed that all O groups possess the same identical H antigen, but different from that of other species of the genus Vibrio. These results confirm the finding of Gardner and Venkatraman. As a result, these authors emphasized again that since cholera and NAG vibrios have similar morphological, physiological and biochemical characteristics as well as a common H antigen they all should be placed in a single species, Vibrio cholerae. These vibrios can be differentiated from each other by their serological behavior and should be subdivided into serotypes according to their O antigens.

Conversion of Ogawa to Inaba and Hikojima serotypes have been recorded as early as 1918 by Kabeshima and 1923 by Nobechei (77). This phenomenon has been particularly studied by Shrivastava and White (1947). Supporting the early observations, they were consistently able to obtain Inaba strains from Ogawa cultures by cultivating them in liquid media containing Ogawa monospecific antiserum, while the reverse transformation from Inaba to Ogawa serotype was not possible under the same conditions, except in certain strains with Hikojima type antigenic properties : Inaba strains in the presence of their homologous antisera normally gave rise to the rough forms. The authors postulated that the Ogawa antigenic composition (factor A + factor B or factor A + factor B + factor C) represents the most complete form of the specific somatic antigen of V. cholerae so far known. This antigenic complex is subject to degradation, which expresses itself serologically as a positive modification, representing the Inaba antigen (factor A + factor C). Further degradation of the antigen will involve the failure of the organism to synthesize the O specific antigen entirely (that is all factors A, B and C). The exposure of the R antigen gives rough serological specificity to the organism.

The above factual observations were re-examined by Bhaskaran and Gor-rill (1957), using the Stocker sloppy-agar technique for the detection of the

variant colonies. Their results were basically the same as above. They stated that Inaba type mutants appeared in Ogawa culture in the presence of Ogawa specific serum (in 24 h) when the original inoculum was greater than 10^7 organisms. These mutants were not isolated if the inoculum was in the neighbourhood of 10^3 organisms although some R forms were still detectable, which may have been due to the higher rate of mutation smooth to rough than that of Ogawa to Inaba. No Ogawa serotype was isolated from Inaba cultures, even when both smooth and rough antisera were added to the Inaba culture while rough forms were consistently isolated. This failure of reversion may have been the result of still lower rate of reverse mutation. When Ogawa strains were mixed with specific antiserum and maintained in the cold to prevent growth, Inaba type was still isolated, which may indicate that in the presence of specific antiserum growth of the culture is not needed for the isolation of Inaba mutants; it is to be assumed that the mutant is present in the Ogawa culture, and the antiserum is simply a selective agent to facilitate their separation. However, ultraviolet irradiation did not cause the appearance of any Inaba type organism in Ogawa cultures without presence of corresponding antiserum.

There have been observations that during epidemics, V. cholerae could be isolated not only from cholera cases, but frequently from healthy individuals as carriers and from the environment as well (19, 26, 36, 37, 50, 89). In the meantime, it seems that the quantity of NAG vibrios in the environment diminishes. Often, in the latter part of cholera epidemics, heterologous serotypes, including rough and NAG forms are isolated. During inter-epidemic period, agglutinable forms of V. cholerae practically disappear from man and environment, except in some individual carriers, and conversely, the number of NAG vibrios increases in the environment (especially in natural waters and water supplies) (77). On the basis of these epidemiological observations, it has been postulated (13, 75, 77, 86) that in natural water, under certain environmental conditions, the cholera vibrio may lose its agglutinability and become a non-agglutinable form. This NAG form will survive in water, during inter-epidemic period, acting as the cholera reservoir in nature. When back to the intestinal tract of man, such an NAG form, under certain biochemical and physiological conditions, once again may revert to agglutinable cholera vibrio; therefore NAG vibrios might be mutant forms of V. cholerae, occupying part of their life cycle in natural waters; they would be responsible for the rise of subsequent epidemics (86, 94). To provide factual evidence to prove this postulate, several experimental works have been performed.

Tomb and Maitra (1927) reported that they had been able to convert agglutinable V. cholerae to non-agglutinable form, and vice-versa, in water tanks and human intestine respectively. Other similar experiments have been done by many authors (11, 12, 14, 75, 86, 94).

Bhattacharji and Bose (1964) have claimed the same success. For the conversion of V. cholerae to NAG form they added some broth culture of Inaba (containing a determined number of the organism) to a quantity of vibrio-free river water samples, possessing a so-called favorable range of pH and a number of coliform bacilli. To these water samples, a small quantity of dry autoclaved soil had also been added. As a control, samples of distilled water were similarly contaminated with V. cholerae. In an overnight period V. cholerae in river water began to lose its agglutinability toward O specific serum, becoming

a NAG vibrio of Heiberg biotype I and II (based on sugar fermentation); it may progressively change to other biotypes depending upon the strain added to water, being in regular smooth, weak agglutinable or rough state. The same strain, in the control water, did not lose its agglutinability and remained unchanged in all respects. In order to show the possibility of reversion, the authors administered a certain number of the various NAG vibrios (isolated either in nature or obtained by the above described method) into the stomach of suckling rabbits under two different conditions using (1) animals with digestive tract of normal pH, and (2) animals in which the pH of the digestive tract was raised by oral administration of alkaline solution. In the animals of the first group, NAG vibrios belonging to Heiberg type I, II and III in their smooth form were pathogenic to the animals; but these vibrios did not undergo any changes in respect of their serological and biochemical behavior. During a series of passages in animals of the second group, the NAG vibrios progressively began to change and, after four to five passages, were fully converted into a typical cholera vibrio, agglutinable with O specific serum.

El-Shawi and Thewaini (1969) have tried to convert several strains of Ogawa El Tor vibrio to non-agglutinable form, by repeating with some variation the first part of Bhattacharji and Bose's experiments, using river water samples sterilized by Millipore filter without addition of any soil. They found that Ogawa El Tor vibrios survived for a period of time in the water samples but without becoming non-agglutinable. Similarly, Biswas and Ganguly (1970) have attempted, without any success, to convert a few NAG vibrios of Heiberg's group I or II to agglutinable form of cholera vibrio. Four NAG strains used in their experiments had antigenic similarity and were cross-agglutinable with each other's antiserum. The authors cultivated the NAG vibrios in human intestinal juice in vitro; after a few days no change in biochemical and serological behavior was observed. By intra-intestinal inoculation of these NAG vibrios in adult or infant rabbits the isolated vibrios after passage through the small intestine did not show any change in their biochemical and serological behavior, compared to those of the original strains. They concluded that it was not possible to confirm the hypothesis of Tomb and Maitra (1926) by their experiments.

It should be noted here that (34, 77), frequently, serological rough variants produce colonies which do not have typical morphological rough characteristics; these colonies are rather smooth in appearance, and they could not be distinguished with certainty by simple inspection. Their definite identification depends on their serological identity which could be demonstrated by using anti-rough serum. These rough forms are not agglutinable with smooth somatic antiserum of V. cholerae; therefore, it is possible that some rough forms have been classified as NAG vibrios, if anti-rough serum was not used to differentiate them from so-called NAG vibrios. It must be also pointed out from the present survey of literature that a spontaneous loss of agglutinability of true cholera vibrio has been reported in stock cultures by some workers (77).

The sero-conversion from Ogawa to Inaba type, and that of smooth to rough form of agglutinable cholera vibrio have been repeatedly reported (6, 7, 79, 97, 102). However, the question as to whether only one or more rough forms do exist still remains to be studied. Previous workers reported that serological roughness of cholera vibrio is not always associated with the morphology of the colony; there is the undeniable possibility that, at least in some cases, the so-called NAG vibrios are likely to be rough forms of V. cholerae. Thus, it is necessary to approach the solution of the problem by studying the immuno-serological relationship between rough and so-called NAG vibrios. The conversion of agglutinable V. cholerae to non-agglutinable form, and reversion of latter to former have faced controversy; perhaps their cause could be due to the fact that certain past works have not been performed under well controlled experimental conditions. With the following studies we hope to bring some light to the solution of this controversy.

* * *

The present studies were aimed

- 1) to obtain various mutants of V. cholerae and to study their biological and immuno-serological behaviors,
- 2) to study the reversibility of mutants thus obtained to the original smooth agglutinable form of V. cholerae,
- 3) to attempt to convert certain NAG vibrios to agglutinable form of cholera vibrio,
- 4) conclusively, based on the data obtained from these studies, to clarify the immuno-serological interrelationships between smooth agglutinable cholera vibrio and its various mutants, including so-called rough form, and furthermore, to explain serological correlation between mutants of cholera and so-called NAG vibrios, which may throw light onto the importance of NAG vibrios in cholera epidemiology.

On the basis of the need to clarify some unsolved problems previously mentioned, the idea of the present studies, with the aims outlined above, was developed. These studies have been encouraged and part of our experiments has been suggested by Yoshikazu Watanabe, M.D., Ph.D., leader of inter-regional cholera team, World Health Organization, Geneva.

II. MATERIAL AND METHODS

1. Media, chemicals and reagents

Media used for this study were Difco products, either in precomposed dehydrated form, or ingredients which were formulated in the laboratory. Components of the various media used are described in appropriate sections. Chemicals and reagents were Merck products of analytical grade.

2. Bacterial strains

The strains used for this study were as follows :

<u>Strain</u>	<u>Type</u>	<u>Supplied by</u>
1. <u>V. cholerae</u>	Classic, Inaba CRC462	Dr J. Gallut
2. <u>V. cholerae</u>	Classic, Ogawa NIH389	Dr J. Gallut
3. <u>V. cholerae</u>	El Tor, Inaba 6973	Dr Y. Watanabe
4. <u>V. cholerae</u>	El Tor, Ogawa 1418	Dr Y. Watanabe
5. NAG vibrio	NAG In-28	Dr J. Gallut
6. NAG vibrio	NAG Og-14	Dr J. Gallut
7. NAG vibrio	NAG N-1	Dr J. Gallut
8. NAG vibrio	NAG 23	Dr J. Gallut
9. NAG vibrio	NAG A-1	Locally isolated
10. NAG vibrio	NAG B-2	Locally isolated

From the above strains, the first two cholerae vibrios and first four of NAG vibrios have been kindly donated by Dr J. Gallut, Cholera Laboratory Service of Pasteur Institute, Paris. Two El Tor vibrios have been kindly supplied by Dr Y. Watanabe, World Health Organization, Geneva. The last two NAG vibrios had been isolated locally from water.

3. Antisera

Commercial antisera were obtained from Difco Laboratories and Pasteur Institute of Paris. Non-commercial sera have been kindly donated by Pasteur Institute and Bacterial Diseases, World Health Organization. Cholera X serum is a factor serum against rough form of V. cholerae (Inaba strain). Other sera were prepared according to the method described later. The potency and specificity of each serum was controlled by a known strain before use.

4. Resorting freeze-dried bacterial strains

Freeze-dried cultures were resorted with 0.5 ml of prewarmed (37°C) sterile nutrient broth, making a uniform heavy suspension. A small quantity of the suspension was serially diluted (10 fold) with nutrient broth; a drop of each dilution was streaked on a prewarmed heart infusion agar plate. The plates were incubated overnight at 37°C.

The purity of the culture was first controlled by appearance of colonies selected from plates containing between ten and fifteen colonies. Several colonies were checked for agglutinability with factor and/or homologous sera by

slide agglutination technique. A suitable individual colony was subcultured and its morphological, biochemical, as well as serological behavior toward anti-smooth and rough sera, once more was controlled. If suitable, the culture was stocked in the medium described below.

5. Maintenance of fresh stock culture

a) Medium

Bacto peptone	2.5 g
Sodium chloride	1.25 g
Bacto agar	1.5 g
Distilled water	250 ml

Ingredients were dissolved by gentle heating and the pH adjusted to 7.4 with 1 N sodium carbonate solution. The medium was then distributed in long narrow tubes, two thirds filled, placing rubber stoppers on with small pieces of string between the tubes and the stoppers and autoclaved for 15 min at 121°C, then cooled in a vertical position; then the strings were removed. Rubber stoppered tubes, after incubation for sterility control, were stored in a refrigerator at 4°C.

b) Inoculation

Prewarmed medium was inoculated by stabbing the loop to a depth of three to four centimeters. Well stoppered tubes were kept at room temperature in a dark place. Prior to its use, the purity of each subculture from the stock was controlled as described above.

6. Biochemical tests

a) Reactions in KIA. Bacto Kligler Iron Agar was chosen to show dextrose utilization (31). It was prepared and employed according to the directions given by The Difco Manual.

b) Oxidase test. Several colonies were covered with 1% aqueous solution of N, N-Dimethyl-P-Phenylendiamine dihydrochloride, which was prepared just before use. The maroon color production was observed in oxidase positive colonies within about one minute. For confirmation, Taxo differentiation discs of BBL were also used according to the prescribed method.

c) Decarboxylase and dehydrolase tests. Each liter of Bacto Decarboxylase medium base contains

Peptone	5.0 g
Yeast extract	3.0 g
Dextrose	1.0 g
Brom cresol purple	0.02 g

4.5 g of this base medium was dissolved in 500 ml of distilled water by gentle heating. To 100 ml portions of the base medium 0.5 g of one of the amino acids, namely l-lysine, l-ornithine and l-arginine was added. The last portion of the base medium was used for control tubes. Their pH was adjusted so that the final reaction of the autoclaved media was 6.5, as shown by the purplish color of the indicator in the media. Each reagent was dispensed in 5 ml quantities into properly marked culture tubes and autoclaved at 121°C for 15 min, cooled, controlled for sterility and stored in the refrigerator.

For use, a set of prewarmed tubes, three amino acids and one control, were inoculated, then overlaid by about 2 ml of sterile mineral oil and incubated at 37°C. The positive results were recorded after 24 h, and the ne-

gative results after three days.

d) Indol test. As medium we used a 10% peptone water, distributed in 5 ml quantities in culture tubes and autoclaved at 121°C for 15 min. After inoculation, the prewarmed tube was incubated overnight at 37°C. 0.5 ml of Kovac's reagent was added to each tube. A dense red color in surface was indicative of positive reaction. Kovac's reagent was made by dissolving 5 g of p-dimethylaminobenzaldehyde in 75 ml of amyl alcohol plus 25 ml of concentrated hydrochloric acid.

e) Mannitol fermentation. A semi-solid mannitol medium was used containing

Bacto peptone	2.5 g
Mannitol	2.5 g
Sodium chloride	1.25 g
Bacto agar	1.0 g
Brom cresol purple	5.0 mg
Distilled water	250 ml

The basic ingredients were dissolved in water by heating it to boiling point, then 1.25 ml of 0.4% solution of brom cresol purple was added. The pH was adjusted to 7.4 and distributed, in 5 ml quantities, in culture tubes, then autoclaved at 121°C for 15 min. The medium was solidified in an upright position. For the test, the tubes were inoculated by stabbing method. Results were read after 24 h of incubation at 37°C.

f) Sugar fermentation for Heiberg biotype. The liquid medium contained

Bacto peptone	2.5 g
Sodium chloride	2.5 g
Brom cresol purple	5.0 mg
Corresponding sugar	2.5 g
Distilled water	250 ml

The corresponding sugar consisted of sucrose, mannose or arabinose (31, 52, 88). The medium was prepared and distributed as for mannitol in a semi-solid medium. The result of inoculated tubes were read after 24 h of incubation; if negative, the tubes were incubated three additional days.

g) Differential tests for classical and El Tor biotypes (5, 48, 84). Polymyxin B sensitivity test was done like the regular antibiotic sensitivity test, using heart infusion agar plate and 50 µg discs of polymyxin B. The result was read after 24 h of incubation.

For the haemolysis test (31), the strain was first grown in heart infusion broth containing 0.85% sodium chloride. Then 1.0 ml of this broth was mixed with 1.0 ml of 1% suspension of washed sheep red blood cells. The mixture was first incubated at 37°C for 4 h, then overnight in the refrigerator. For reagent control 1.0 ml of sheep cell suspension was mixed with 1.0 ml of heart infusion broth. Next day the haemolysis was observed in supernatant fluid after 10 min of centrifugation at 2500 rpm.

For chicken red blood cell agglutination test (31, 35) one drop of a 2% washed saline suspension of cells, on a slide, was mixed with one drop of heavy bacterial saline suspension. If positive, agglutination of red cells appeared within about 1 min.

7. Preparation of antisera

a) Preparation and standardization of antigen. Large plates of heart infusion

agar were heavily inoculated from 5 to 6 h heart infusion broth cultures. After 18 h of incubation at 37°C the cell mass was harvested with sterile physiological saline solution. Then the cell suspension was heated in boiling water bath for 2 h.

Standard curve was prepared according to the McFarland scale for standardization of bacterial suspension, using Coleman Junior Spectrophotometer. The concentration of boiled antigen was standardized, against the standard curve, so that each ml of antigen suspension contained approximately 2.0×10^9 cells.

b) Immunization. For each type of antigen a minimum of two rabbits, each weighing between 3 to 4 kg, were used. Pre-immunization blood samples were obtained from each rabbit for control. Adapting a light immunization technique, on the first day 0.5 ml of freshly prepared antigen was injected intravenously. After four days the second injection, consisting of 1.0 ml antigen, was also intravenously given. Bleeding was done one week after second injection. By light immunization method moderate to rather high titre antisera were generally obtained. It was noticed that in cases with low titre, a booster injection after a short interval did not give significant rise of titre, unless the boosters were repeated several times within a few days interval. But since the aim was to produce antisera with a minimum of cross-agglutinating antibodies, hyper-immunization was avoided.

c) Separation and preservation of antisera. Animals were bled by cardiac puncture, and the blood of each rabbit was collected separately in chemically clean and sterile 250 ml screw-capped bottles. The bottles were placed, in a slant position, on the table surface, at room temperature for 30 min, until blood clotted, then overnight in a refrigerator. Next day, clear sera were collected, each sample separately, in large centrifuge tubes. Then the surface of the clots were cut, at several points, with a sharp sterile blade. The clots were replaced in refrigerator for several hours and were centrifuged directly at 3000 rpm for 30 min. If sufficiently clear, these additional quantities of sera were transferred to the corresponding original serum bottles. Once again, the original serum bottles were centrifuged at 3000 rpm for 30 min and cell free, clear sera were transferred into sterile containers. To each volume of serum, an equal quantity of sterile, reagent grade glycerol was added and maintained in the refrigerator.

d) Titration of antisera. The titre of each 50% glycerinated serum was determined by slide and tube agglutination methods, as described below, using homologous antigen. If the titres of each individual serum from corresponding antigen were sufficiently high, the sera were pooled. The pooled serum was once more titrated then distributed into 10 ml containers and stored in the refrigerator.

8. Agglutination tests

a) Preparation of antigen. The bacterial strain was cultured, harvested and heated exactly as described before in immunization section (7b). After heating, the cell suspension was centrifuged at 5000 rpm for 30 min. The supernatant was discarded and the cell pack was resuspended in phosphate buffered saline (pH 7.4). Using a standard curve as before, the antigen suspension was standardized. The antigen for the tube agglutination tests contained 1.0×10^9 cells per ml; the antigen for slide agglutination was five times more concentrated.

b) Slide agglutination. For quantitative test the serum was serially diluted with physiological saline in small tubes. On properly divided slides, one drop of freshly prepared antigen suspension for slide test, using a 20 G needle and a 1 ml syringe, was placed with one drop of serially diluted antiserum; a saline antigen control also was used. After mixing the slides were rotated gently for 1 min. Grading the agglutination results from the most complete (++++) to the least partial (W), the highest dilution of the serum which gave at least a (+++) agglutination was recorded as the final slide titre. Each test was performed in duplicate.

For the routine qualitative slide agglutination test, a small quantity of each serum was diluted, so that it would be four times more concentrated than the final slide titre. For example, a serum with slide titre of 1 : 32 (compensating for 50% glycerine added) was diluted to a final volume of 1 : 8 for routine slide agglutination test. As a vehicle, a 1% bovine serum albumin in phosphate-buffer saline (pH 7.2) was used ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 g; KH_2PO_4 , 0.2 g per litre of 0.85% saline). To the final product 0.01% merthiolate was added as a preservative, and serum was kept in a small dropper bottle in the refrigerator for routine use.

Qualitative slide agglutination tests, for identification of prospective antigens, were also performed according to the basic technique described above. For the sake of uniformity, reproducible and comparable results, boiled standardized antigen, as described before (8a), has been used in all quantitative and qualitative slide agglutination tests, unless indicated otherwise.

c) Tube agglutination. From a 1 : 10 diluted serum (compensating for 50% glycerol added), a 2-fold serial dilution was made. Using 0.25 ml of serially diluted serum, and 0.25 ml of antigen suspension the tube test was set up in duplicate, including antigen control. Tubes were incubated overnight in 52°C water bath. In cross-agglutination tests, control system consisted of antigen suspension in saline for negative control, and the corresponding serum and antigen for positive control.

Considering the first tube as the final dilution of 1 : 20, the results were observed and recorded the next day. The agglutination as in the slide test was graded from the most complete (++++) to the least partial (W) reactions, and the reciprocal of the highest dilution which gave at least (++) reaction was considered the titre of the serum to the corresponding antigen.

At the beginning of the experiments, after reading the results following 52°C incubation time, a second reading was done after placing tubes in the refrigerator overnight. Since no definite consistent change in titre was observed, the refrigeration was discontinued in the proceeding experiments.

d) Agglutinin absorption. In order to test the ability of a strain to absorb a given antibody, or to detect antibody by absorption method, it was necessary to determine the exact quantity of the antigen needed for absorption.

First, tube agglutination titre of the serum with corresponding antigen was determined; on the basis of this titre, the quantity of antigen needed for absorption of the respective antibody in 1 ml of serum was calculated. Example : the titre of the serum is 1 : 640, i. e. 0.25 ml of 640 times diluted serum is needed to agglutinate 0.25 ml of an antigen suspension with concentration of 1.0×10^9 cells per ml; therefore the quantity of cells needed for each ml of undiluted

serum (or two ml of 50% glycerinated serum) would be :

$$\frac{1.0 \times 10^9 \times 640}{0.25 \times 4} = 6.4 \times 10^{11} \text{ cells/ml of serum}$$

The calculated amount of packed cells which was prepared for absorption of each ml of serum was suspended in 1 ml of saline, so that it could be drawn easily into a pipette.

For absorption, 0.5 ml of antigen was added to 1 ml of serum; the mixture was incubated at 52°C water bath for 2 h, then centrifuged at 10,000 rpm for 30 min. The serum was transferred to another tube and the remaining 0.5 ml of antigen was added. The process of incubation and centrifugation was repeated once more, and the absorbed serum was separated. A control consisting of serum and saline mixture was always made and treated in the same manner as serum-antigen mixture. The agglutinability of prepared absorbed serum with the corresponding antigen was checked again by the tube agglutination method before its use.

9. Isolation of motile forms

a) Medium. Semi-solid medium of Edwards (1964) for isolation of motile strains was used :

Bacto peptone	10 g
Bacto gelatin	80 g
Bacto meat (or beef) extract	3 g
Bacto agar	4 g
Sodium chloride	5 g
Distilled water	1 l

Gelatin and sodium chloride were dissolved by gentle heating in 600 ml of water, and the remaining ingredients in 400 ml of water; then the two solutions were mixed. The pH was adjusted to 7.2 with 1 N sodium carbonate solution and the medium was autoclaved at 121°C for 15 min. After cooling to about 50 to 60°C, the medium was distributed in regular size Petri-dishes, and kept in a closed container in the refrigerator.

b) Technique. Four isolated colonies of a pure culture were subcultured, each in a tube containing 2 ml of heart infusion broth, and incubated for 6 to 8 h at 37°C.

Four Edwards plates were prewarmed for 30 min at 37°C and each plate was inoculated, using a Pasteur pipette, with a few drops of the above broth cultures on one spot near the edge of the agar surface of the plate. Sterile filter paper was placed inside the Petri-dish cover in order to absorb the moisture produced during the incubation period. The plates were incubated at 37°C.

The growth in these plates is in semi-circular form. The non-motile bacteria will grow rather locally, while the flagellated forms progressively grow outward. The extent of the growth within 24 h depends upon the motility of the culture; normally after 3 to 4 passages on Edwards plates, strongly motile strains were obtained. Therefore, after the first 24 h, growth from the edge of the most progressed section of the semi-circle was transferred to a new prewarmed Edwards plate, and the passage was continued in this manner, until a highly motile strain was obtained.

10. Isolation of mutants

a) Medium. For this part of the experiment, a low nutritive, semi-solid medium was used. It contained per litre the following ingredients :

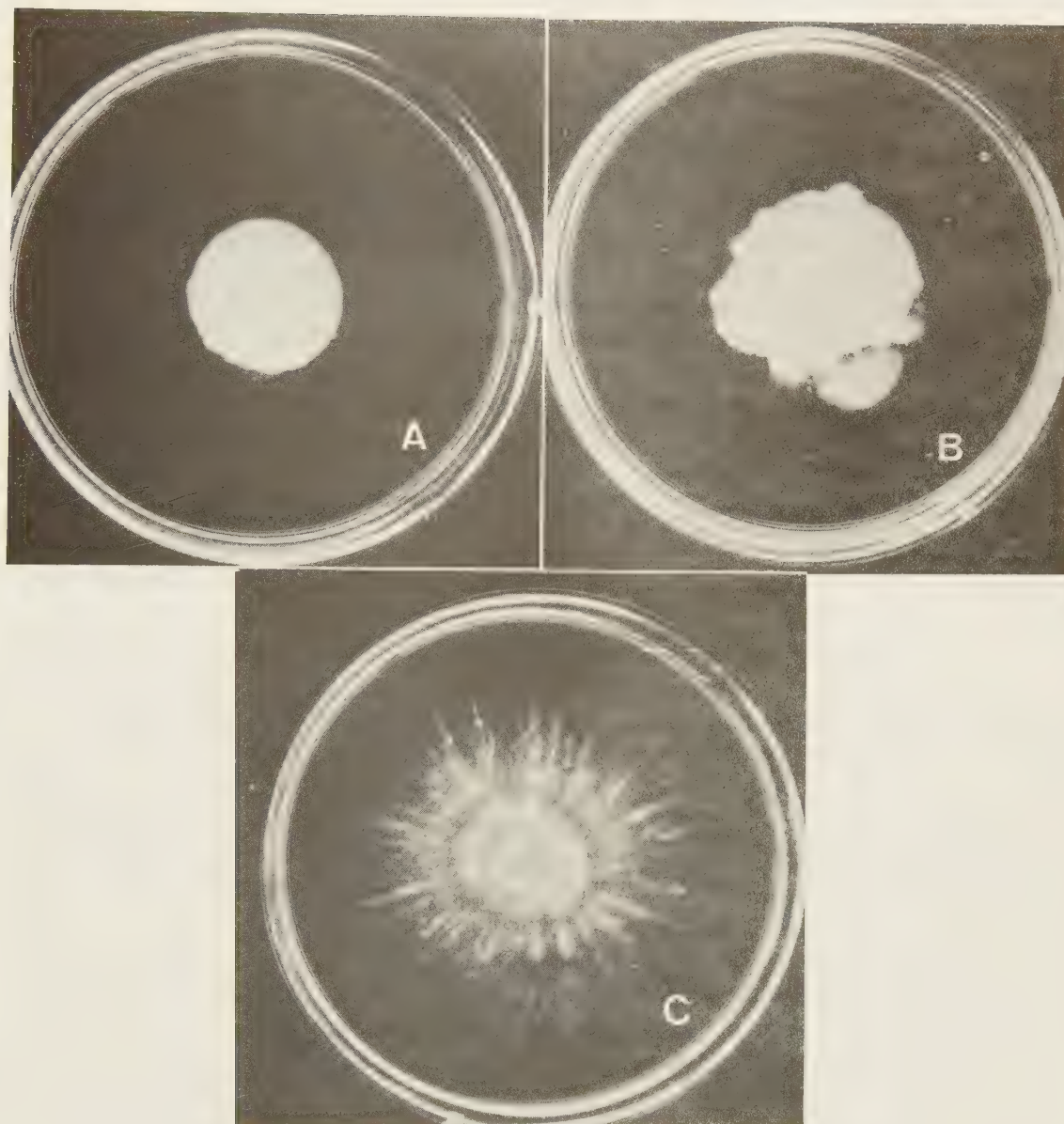
Infusion, from beef heart	250 g
Tryptose	5 g
Sodium chloride	5 g
Bacto agar	4 g

Preparation of the medium: 12.5 g of Difco heart infusion broth, 2.5 g of sodium chloride and 4.0 g of Bacto agar were dissolved in 1 l of distilled water. The mixture was gently heated to boiling point, cooled to 50°C, and its pH adjusted to 7.2 with 1 N sodium carbonate solution. It was distributed in 50 ml quantities, into small flasks, autoclaved at 121°C for 15 min, cooled and kept in the refrigerator. At the time of use, a sufficient quantity was remelted in boiling water bath and cooled to about 50°C. Then serum and control plates were prepared using small Petri-dishes of 50 mm diameter. The quantity of medium used was about 7 to 8 ml per plate.

For these plates we used 50% glycerinated and pre-titrated sera, as described before. The quantity of antiserum was 1% of 1 : 320 base titre of pure serum : 2 ml of 50% glycerinated serum with a titre of 1 : 320 was added to each 100 ml of the medium (this quantity is referred to as 3.2 agglutinin units per ml of medium). If, for example, the titre of serum was 1 : 640, only 1 ml, and if the titre of serum was only 1 : 160, 4 ml of 50% glycerinated serum were used per 100 ml in order to get a consistent quantity of 3.2 agglutinin units per ml of medium. It was thought that in this manner the effect of the antisera as a selective inhibitor could be kept constant. However, in occasional cases, the use of more serum was needed for isolation of mutants. Control plates contained only the basic medium. Prepared plates were stored in closed containers in the refrigerator.

b) Principles of the technique. A motile strain in semi-solid medium will normally grow progressively and within several hours to overnight cover the entire surface of the plate. The presence of homologous O antiserum causes the adherence and aggregation of the cells, giving a massive local growth more or less similar to a colony on solid media, but in giant size. If in seeded bacterial population and their subsequent offspring, some mutants are present with such a somatic antigenic structure that the presence of the antiserum could not cause them to aggregate, they will have the liberty of growing outward; they will be separated from the main body of the bacterial population and easily selected. This phenomenon has been termed 'bulging of mutant', and the technique as 'bulging method'.

Control plates, normally within overnight incubation, gave a uniform growth over the entire surface of the plate. Four basic types of growth were observed on serum plates : (i) if the antiserum present in the plate could not act as a selective inhibitor, as it was anticipated, the bacteria grew over the entire surface of the plate as in the control case; (ii) if antiserum was effective on the seeded bacteria, and no mutant present in bacterial population, the growth appeared in a small heavy circular form, which became thicker and larger over the period of incubation, usually with a thinner outer ring; (iii) the growth of the main bacterial population, whose movement was inhibited by antiserum,



- A (ii) Massive circular growth without any bulging.
B (iii) Massive circular growth from which a mutant has bulged out.
C (iv) Radiated type of growth.

Figure 1. Different forms of growth on serum plates.

was similar to the above form, but the mutants present in bacterial population (whose movement could not be effected by the antiserum present in the plate) bulged out, in one or several points, from the main ring and progressively grew outward; (iv) we observed a radiated type of growth, similar to the burst of a fire cracker, which progressively grew out on the surface of the plate; the cause of this phenomenon has not been clearly understood. Figure 1 shows different forms of growth on serum plates. It should also be mentioned here that the use of 3.2 agglutinin units per ml of medium was the best concentration for isolation of most mutants with diversified O antigenic behavior.

c) Technique. From each of the four Edwards plates produced from the same strain, 4 tubes of prewarmed heart infusion broth were heavily inoculated. The tubes were incubated at 37°C for 18 h. From these broth cultures, the appropriate serum and control plates were inoculated, serum plates in duplicate, by placing a few drops of well mixed broth culture in the center of each plate. In this manner each original strain was represented in 8 serum plates and 4 control plates. All plates were incubated at 37°C.

The plates were kept at 37°C for a few days, and each day they were checked for appearance of bulging. If bulging occurred, material from the outermost part of the bulging was transferred to a second, and eventually to a third serum plate.

d) Control of isolated mutant. From the last serum plate, by serial dilution technique, a pure culture was obtained. Then its growth behavior in the serum plate, its biochemical reactions and serological characteristics were controlled.

For growth behavior, four semi-solid plates were taken, 2 for control and 2 with antisera of parent strain, which was used before. One pair of the control and serum plates were inoculated with the parent strain and the other pair with the new mutant strain. All plates were incubated only 12 h at 37°C. As was expected the control plates and serum plate containing the mutant strain had uniform growth over the entire surface of the plate, while serum plate containing the parent strain gave only a small ring of massive growth. The biochemical and serological tests have been described in appropriate sections (6, 8b, c). The isolated mutant was stocked for future work.

11. Preparation of lipopolysaccharides (LPS)

a) Cultivation and harvesting of bacterial cells. For each bacterial strain, 15 large plates, each containing 100 ml of Bacto heart infusion agar, were prepared. These plates were heavily inoculated with a six hour Bacto heart infusion broth culture. The plates were incubated overnight at 37°C. Next day, the cell mass was harvested by washing the surface of the plates with sterile distilled water, so that the final volume was 125 ml.

b) Extraction and purification of LPS. The technique used for extraction was basically the warm phenol-water extraction method (18, 24, 39, 41, 61, 71, 74, 98, 100). 125 g of phenol were slowly heated to melting point in a water bath. The cell mass suspension was also warmed to about 50°C, then melted phenol was added to the cell mass. The mixture was gently stirred with a glass rod, while gradually increasing the temperature to 65°C, which brought the mixture to one homogenous phase. The mixture was kept at this temperature for 20 min with constant stirring. Then the mixture was cooled to 5°C, and

centrifuged at 8500 rpm for 30 min, while kept at 5°C. After centrifugation, phenol-water mixture was separated into two phases : the phenol phase on the bottom contained protein material; the water phase on the top contained lipopolysaccharides and nucleic acids with a thick white fluffy layer between the two phases.

The water phase was carefully transferred to another container, and dialyzed in a cellophane bag for 72 h against distilled water in the refrigerator. The dialyzed solution was centrifuged at 8500 rpm for 30 min to separate non-soluble material, if any. The clear solution was transferred to a wide-mouth container, and concentrated to about 10 to 15 ml under vacuum.

To this concentrated aqueous phase, ten volumes of chilled absolute ethyl alcohol, and a trace amount of sodium acetate were added, and after mixing well, it was placed overnight in the refrigerator, until LPS were totally precipitated. Then the mixture was centrifuged at 12,000 g for 15 min, at 5°C. The LPS formed a gel packed in the bottom of the tube; the supernatant was decanted. The precipitate was resuspended, and washed in chilled ethyl alcohol several times, each time, centrifuging and decanting the alcohol. This process washed off most of the nucleic acids present in LPS gel, so that the last supernatant practically did not absorb any light at 2600 Å when tested in U. V. spectrophotometer.

c) Preservation of LPS. Gel type LPS precipitate was dried from the alcohol, dissolved in a small quantity of water, and an equal volume of sterile neutral glycerol was added to it. The mixture was an opalescent solution with moderate viscosity. The precipitating ability of this LPS was checked, and its concentration adjusted as it will be described below. It was distributed, in small quantities, into small tubes and conserved in a deep-freezer (-20°C).

12. Immunodiffusion tests

a) Antigen-antibody system. The basic technique chosen for precipitation tests was slide double diffusion method of Ouchterlony with agarose as a gelling agent (27, 49). As antigens we used the prepared LPS of various strains. Two kinds of antisera were considered in these tests : (i) the original undiluted, or 50% glycerinated sera, which were prepared by light immunization method for the purpose of agglutination tests, showed in most cases to be satisfactory for precipitation tests; (ii) the second type of serum was an antiserum prepared against whole cell lysate (WCL) of smooth V. cholerae strains.

For preparation of WCL, a quantity of cell mass was prepared as it was described for LPS elaboration. The cell mass was washed a few times with saline and resuspended in 15 ml of distilled water in a plastic container. The lysate was prepared by placing the suspension at 56°C for 6 h and then transferring it for 6 h into a deep-freezer at -70°C; this process was repeated 15 times until the cells were totally lysed. For preparation of anti-WCL serum, the concentration of antigen was adjusted so that it contained about 900 mg % of nitrogen. The rabbits were injected subcutaneously, each with a mixture of 1 ml antigen plus 1 ml of Bacto complete Freund's adjuvant; the second injection was given after one week, and the third after three weeks. The animals were bled on the 25th day after the first injection.

Determination of the optimal concentration of antigen

A serial 2-fold dilution of antigen was made. With each dilution of antigen and constant quantity of homologous antiserum we prepared one slide for double

diffusion test in gel. We used also at first a tube precipitation method in liquid at 37°C, using constant quantity of antiserum and serially diluted antigen, but the method did not give uniform consistent results and was discontinued.

b) Preparation of slides. Buffered-saline of pH 7.4 which was used for preparation of the gel, contained

Sodium barbital	6.98 g
Sodium chloride	8.0 g
1 N Hydrochloric acid	27.0 ml
Distilled water	to 1000 ml

One gram of agarose was dissolved in 100 ml of the above buffer, by heating it in a boiling water bath, then 1 ml of 0.01% merthiolate was added as a preservative. The mixture was distributed in 25 ml quantities, and kept in the refrigerator.

Alcohol washed and cleaned glass slides (46 x 76 mm) first were covered with a thin layer of a 0.2% aqueous solution of agarose, and were dried in a 37°C incubator. This thin layer secured adherence of the subsequent layer of the agarose to the slide. A sufficient quantity of 1% agarose in buffered-saline was melted in a boiling water bath, and was cooled to about 50°C. Each slide was covered with 4.0 ml of agarose, which gave a layer of about 1.5 mm thickness. The slides, thus prepared, were placed in a moist chamber in the refrigerator to be well solidified (the moist chamber consisted of a large Petri-dish with wet filter paper and two supporting rods on the bottom, and a filter paper inside the cover).

With the aid of a pattern which was drawn on paper and placed underneath the slide, the wells were cut into agarose with a metal puncher, and excess of agarose was removed from the wells with the end of a Pasteur pipette which was attached to a suction pump. The slides were placed again into the moist chamber, sealed and kept in the refrigerator for future use.

c) Set up of the test. With the aid of capillary pipettes each well was filled with the appropriate antigen or antiserum with special care that no air bubbles would be trapped in the wells. The slides were placed back into the moist chamber and kept in the refrigerator. They were controlled for precipitation lines at intervals of three or four days. The best results, with a maximum number of lines, were usually obtained within about a week. At the end of this period, slides were washed in saline for 24 h, with frequent change of the solution. Finally they were washed in distilled water for 1 min; a moistened blotting paper was placed on the top of the agarose and the slides were placed in 37°C incubator until the next day, then the filter paper was removed from the top of the dried agarose. The slides were ready for staining.

d) Staining. Just before staining the dried slides were washed in tap water for a few seconds, then stained for 15 min in coomassie brilliant blue solution. After decoloration for just a few minutes depending on the color density of the slides, the slides were washed and dried. Each litre of staining solution contained 2.5 g coomassie brilliant blue, 454 ml methanol, 92 ml acetic acid and distilled water q. s. ad 1000 ml. Decolorizing agent contained 700 ml methanol, 10 ml acetic acid and 300 ml distilled water.

III. RESULTS

A. ISOLATION AND CHARACTERIZATION OF VARIOUS MUTANTS OF CHOLERA AND NAG VIBRIOS

1. Notes on smooth parent strains

Cultural and biochemical characteristics. For isolation of different classes of mutants, four strains of V. cholerae and six strains of NAG vibrios, which were described in section II. 2. have been used. The cultural characteristics of four V. cholerae strains were basically the same. The first three NAG vibrios : NAG In-28, NAG Og-14, NAG N-1, having the cultural characteristics of a semi-rough form, were similar to each other; but these characteristics were different from those of V. cholerae, as well as those of the three other vibrios, namely NAG 23, NAG A-1 and NAG B-2 (Table I).

All four strains of V. cholerae tested for their biochemical behavior gave identical reactions (Table II). The following tests were also performed to show the differential characters of the two biotypes :

- Classic strains were non-haemolytic, sensitive to polymyxin B (50 µg) and gave a negative result in chicken-cell haemagglutination test;
- El Tor strains were haemolytic, resistant to polymyxin B (50 µg) and agglutinated chicken-red blood cells.

The biochemical reactions of six NAG vibrios, which are also shown in Table II, were identical to those of V. cholerae, except NAG B-2 which belongs to Heiberg group II.

Serological behavior. The serological behavior of these strains was checked by slide and tube agglutination, with cholera polyvalent, Inaba and Ogawa factor sera, homologous sera and cholera X (anti-rough) serum. The corresponding antisera of V. cholerae strains gave agglutination (titre range of 1 : 320 to 1 : 640) with homologous antigen as well as with the antigen of the other similar serotype and partial cross-agglutination of lower titre with opposite serotype. The antisera of NAG vibrios gave high titre agglutination only with their own homologous antigen. Agglutination results of these vibrios are shown in Table III.

Stability of the strains. The strains proved to be stable in stabbed stock cultures, which were controlled periodically within two years.

2. Mutants of cholera vibrio strains

2. 1. Isolation of typical rough mutants

Typical rough mutants, which have been termed RI, were isolated from previously named Inaba smooth strains in the presence of their corresponding S antiserum and from Ogawa strains in the presence of S Ogawa plus S Inaba

Table I

Cultural characteristics of smooth parent strains of the vibrios used in these studies

Strains Characters	<u>Vibrio cholerae</u>	NAG vibrios In-28, 0g-14, N-1	NAG vibrios 23, A-1, B-2
Colony appearance on solid media	circular, raised, 1-2 mm in diameter, semitrans- parent, smooth, soft, easily suspended	circular, rather raised 1-2 mm in diameter, semitransparent, smooth type, granular, easily suspended	circular, raised, 2-3 mm in diameter, semi- transparent, smooth and rather mucoid, easily suspended
Growth appearance in liquid media	homogeneous turbidity, no sediment, no surface pellicle	moderate turbidity, moderate sediment, heavy surface pellicle	homogeneous turbidity, no sediment, light surface pellicle

Table II

Biochemical reactions of smooth parent strains
of the vibrios used in these studies

Organisms Tests		Cholera vibrio	NAG vibrios In-28, Og-14, N-1, 23, A-1	NAG vibrio B-2
KIA	Slant	Ak	Ak	Ak
	Butt	A	A	A
	Gas	-	-	-
	H ₂ S	-	-	-
Oxidase		+	+	+
Arginine dehydrolase		-	-	-
Lysine decarboxylase		+	+	+
Ornithine decarboxylase		+	+	+
Indol		+	+	+
Mannitol		+	+	+
Heiberg fermentation	Mannose	+	+	-
	Sucrose	+	+	+
	Arabinose	-	-	-

Key: + Positive reaction
Ak Alkaline

- Negative reaction
A Acid

Table III

Agglutination reactions of smooth parent strains of the vibrios used in these studies

Strains Antisera										
	Inaba CRC462	Inaba 6973 (El Tor)	Ogawa NIH389	Ogawa 1418 (El Tor)	NAG In-28	NAG Og-14	NAG N-1	NAG 23	NAG A-1	NAG B-2
Cholera polyvalent	+++ 320	+++ 640	+++ 640	+++ 640	+ 80	+ 40	- 20	- 0	- 0	- 0
Inaba factor serum	+++ 320	+++ 320	- 0	- 0	W 40	+ 40	- 0	- 0	- 0	- 0
Ogawa factor serum	- 0	- 0	+++ 320	+++ 320	- 0	- 20	- 0	- 0	- 0	- 0
Corresponding anti-S serum	+++ 320	+++ 640	+++ 640	+++ 320	+++ 640	+++ 1280	+++ 640	+++ 640	++ 320	+++ 320
Anti-rough serum (Cholera X)	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0

Slide agglutination results are graded from the most complete(++++) to the least partial(W) reaction. The figures give the titres of the tube agglutination, 0 indicates a titre less than 1:20.

antisera. Bulging of mutants in serum plates appeared within 24 to 48 h.

The colonies were generally larger than smooth form (about 3 mm in diameter), semi-transparent, flat or slightly raised, dry but smooth appearing, so that they were not, at the first sight, distinguishable from the smooth form. Only colonies of RI-6973 had rough appearance. In liquid medium, they gave a clear supernatant, with moderate to heavy sediment. RI-6973 produced surface pellicle, three others produced none. The cultural characteristics of these rough mutants are shown in Table V. As far as biochemical behavior was concerned, no changes occurred as compared with those of parent smooth strains.

Serological behavior. The agglutination reactions of these four rough strains with their homologous and cholera X (anti-rough), as well as smooth sera are shown in Table IV. Their cross-agglutination behavior with other antisera is also shown in Tables XI, XII and XVII. These strains were not strongly stable. In stabbed stock cultures kept at room temperature (16 to 25°C), within one and a half years, a few of them showed reversion toward S Inaba serotype. Under given conditions and length of time this reversion was not unexpected.

2. 2. Mutants isolated from RI strains in the presence of anti-RI sera

The four previously isolated typical rough forms of *V. cholerae* were cultured in the presence of their corresponding homologous RI antiserum. Basically, they all had a strong tendency to shift back to the smooth Inaba form, and the latter were frequently isolated. In addition, two forms of mutants (RIIa and RIIf) were isolated from them :

(i) The first form, termed RIIa is derived from RI-462, RI-6973 and RI-389; it appeared serologically to be similar to the original smooth form, but showed cultural characteristics of a semi-rough strain. Their biochemical characteristics, comparing with S and RI parent strains, remained unchanged.

In fact, the serological behavior of these mutants was somehow different. The mutants were agglutinable by corresponding antiserum of S parent strain, as well as Inaba or Ogawa factor serum, depending on their serotype, but weaker than their smooth parent strains. An absorption test on a sample of anti-S 462 serum, showed that the quantity of RIIa 462 cells needed to absorb Inaba factor was about four times more than that of S 462 cells. Anti-RIIa-462 serum, having a titre of 1 : 320 with its homologous antigen, as well as anti-RIIa-6973 gave the same titre with S parent cells, but only a titre of 1 : 80 with RI cells. Anti-RIIa-389 serum also gave similar reactions with corresponding antigens. The serological behavior of this kind of mutant, compared to those of parent strain and other mutants is shown in Table XII. This form of mutant, because of the above properties, has been considered as semi-rough (SR) form. Table V shows the characteristics of SR form, compared to the S parent strain, as well as rough forms. These strains were rather unstable; kept in stabbed stock cultures at room temperature, they mutate either to complete smooth or rough form within a few months.

(ii) The mutant isolated from RI-1418 showed cultural characteristics similar to the above form (SR) mutant, without any change in biochemical reactions; but its serological behavior was different : it was not agglutinable to an acceptable degree neither by S nor RI antisera. Its behavior was similar to mutants termed RIIf, which will be described later.

Table IV
Agglutination reactions of RI forms of cholera vibrios

Antisera \ Strains	RI of Inaba CRC462	RI of Inaba 6973	RI of Ogawa NIH389	RI of Ogawa 1418
Corresponding RI sera	+++ (320)	++++ (640)	+++ (320)	++++ (640)
Anti-rough serum (Cholera X)	++ (160)	+++ (160)	++ (160)	++ (160)
Corresponding S sera of parent strain	W(40)	-(0)	W(40)	W(80)
Inaba factor serum	-(0)	-(0)	-(0)	-(0)
Ogawa factor serum	-(0)	-(0)	-(0)	-(0)

Slide agglutination results are graded from the most complete (++++) to the least partial (W) reaction. The figures in the parenthesis give the titres of the tube agglutination. 0 indicates a titre less than 1:20.

Table V

Characteristics of rough and semi-rough mutants of cholera vibrio compared with those of smooth parent strains

Strains Characters	S parent strain	SR form	RI form
Colony appearance on solid media	circular, raised, 1-2 mm in diameter, semi- transparent, smooth, soft, easily suspended	circular, rather raised, 1-2 mm in diameter, semi- transparent, smooth type, granular, easily suspended	generally smooth appearing, but flat, larger than S form, dry and granular, generally gives good suspension, may be auto-agglutinable in saline
Growth appearance in liquid media	homogeneous turbid- ity, no sediment	moderate turbidity, moderate sediment	clear supernatant, heavy sediment
Slide agglutina- tion with: corresponding anti-S serum corresponding anti-SR serum corresponding anti-RI serum	++++ ++++ - to W	++ ++++ - to W	W W to + ++++

Slide agglutination results graded from the most complete(++++)to the least partial(W) reaction. - indicates no agglutination.

2. 3. Attempts to isolate mutant from smooth strains in the presence of anti (S+RI) sera

Smooth form of all previously named V. cholerae strains were cultured in the presence of their corresponding anti (S+R) sera. In all cases growth was in small massive form, with no progressive movement or bulging. Frequent subculture, from these serum plates, showed only the presence of originally seeded smooth strains, and no new mutants were isolated.

2. 4. Mutants isolated from RI strains in the presence of anti (S+RI) sera

This experiment was also performed with all four rough strains of V. cholerae, using serum plates containing corresponding anti (S+RI) sera. The mutants derived from these strains were, basically, in four categories.

(i) The first category consists of mutants with the cultural, biochemical and serological characteristics of the smooth Inaba type. These smooth type mutants seemed to be able to pass antibody barriers after several days of incubation. They either grew as bulging type or showed a progressive ring type. It is to be pointed out here that, because of the unique behavior, the purity of rough strains was rechecked, making sure that the plates were inoculated with pure rough culture. On the other hand, when the quantity of sera was increased beyond 3.2 agglutinin units of each anti-S and anti-RI serum per ml of media, the growth was inhibited. This observation, which could be regarded as a shift back to the smooth Inaba form, was rather frequent in all cases; it indicates a high frequency of reversion from RI to S form.

(ii) By the studying of the above mentioned mutant a few isolates from Inaba origin rough forms showed the cultural, biochemical and serological characters of SR mutants which were described earlier.

(iii) From plates seeded with RI of Ogawa 389, in addition to the smooth Inaba serotype, a mutant was also isolated, which had the cultural, biochemical and serological characteristics similar to those of Hikojima serotype strain.

(iv) In very few serum plates containing corresponding anti (S+RI) sera, which were seeded with RI-462 strain, a new class of mutant was encountered. Because of the unique serological behavior of this mutant, which was termed RI**IIb**, an attempt was made to separate similar forms from other strains. From RI-6973 strain, a similar mutant bulged out in serum plates containing a double amount (6.4 agglutinin units per ml) of both anti S and RI sera. From RI-389 and RI-1418 strains, a similar mutant bulged out when serum plates contained, in addition to rough serum, both Ogawa and Inaba antisera at a level of 6.4 agglutinin units per ml. These mutants normally bulged out in about 72 h. Their cultural characteristics on solid and in liquid media were similar to those of a semi-rough strain. RI**IIb**-462 and RI**IIb**-1418, in liquid media, produced light surface pellicle, while two others did not, when incubated for 24 h at 37°C. No change was observed in respect to the biochemical reactions compared to those of parent strains.

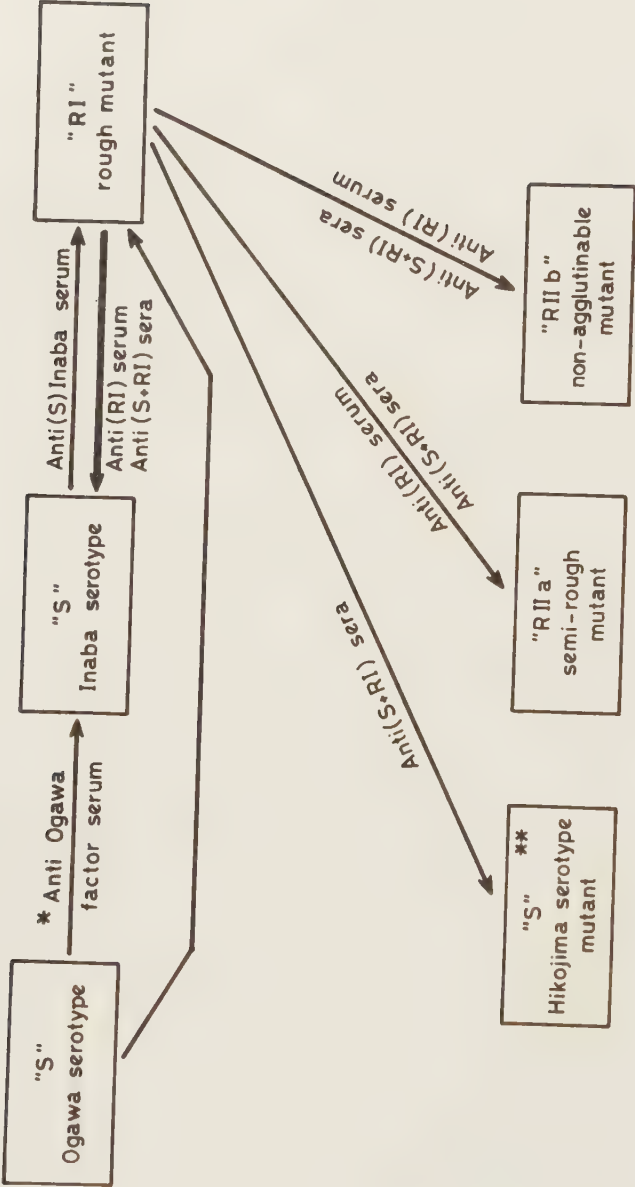
Serological behavior. By slide agglutination technique these mutants were not agglutinable to an acceptable degree either by cholera polyvalent, Inaba and Ogawa factor sera, or by corresponding anti-S or -RI sera. The results of tube agglutination confirmed those of the slide method. Table VI shows agglutinability of these mutants compared to S parent strains. The antigenic analysis of

Table VI

Serological behavior of RIIB mutants compared with those of smooth parent strains

Strains Antisera	RIIB 462 (Inaba origin)	RIIB 6973 (Inaba origin)	RIIB 389 (Ogawa origin)	RIIB 1418 (Ogawa origin)	Smooth Inaba	Smooth Ogawa
	+	+	W	W	+++	+++
Cholera polyvalent	40	40	40	40	320	640
Inaba factor serum	W	+	W	+	+++	-
	40	40	40	40	320	0
Ogawa factor serum	-	-	-	-	-	+++
	0	0	0	0	0	320
Corresponding anti-RI serum	±	W	±	W	-	-
	20	40	20	40	0	0
Homologous RIIB serum	++++	++++	++++	++++	W	W
	320	640	320	640	40	40

Slide agglutination results are graded from the most complete(++++), to the least partial(W), indicates a doubtful reaction. The figures give the titres of the tube agglutination.



* This conversion was not performed in these studies but it has been demonstrated by several investigators (7,77).
** Obtained from RI Ogawa 389 only

SCHEMATIC PRESENTATION OF MUTANTS ISOLATED FROM CHOLERA VIBRIO

Figure 2

these mutants and their relationships to other mutants will be discussed later. It should be mentioned here that cell suspensions of these strains in saline were stable, giving a uniform but slightly granular suspension. These strains were rather unstable; in stabbed stock cultures kept at room temperature their characters were changed within a few months. They became more agglutinable with cholera polyvalent, Inaba factor serum, as well as with anti-RI and anti-RIIa sera, each to a different degree, but all partially. The mutants isolated from smooth and rough forms of V. cholerae, are shown schematically in Figure 2.

3. Mutants of RIIb strains

As seen before RIIb strains which were derived from RI strains do not agglutinate with O smooth or rough antisera within an acceptable degree and could be considered as non-agglutinable forms. The antigenic analysis of these strains and their relationships to other strains will be discussed later.

The following experiments, which are described in the next four sections, were performed in order (i) to observe the possibility and the manner of the reversion of RIIb strains toward typical rough, as well as toward smooth agglutinable forms of V. cholerae, and (ii) to isolate prospective new mutants. In these experiments, RIIb strains, originated from both Inaba and Ogawa serotypes were used. The growth behavior was observed in serum plates containing a different combination of sera, namely anti (RIIb), (RIIb+S), (RIIb+RI) and (RIIb+S+RI) sera. For each strain, the homologous anti-RIIb serum, as well as anti-S and anti-RI of the corresponding parent strains were used. The mutants isolated from these RIIb strains are schematically presented in Figure 3, and their serological behavior in Table VII.

3. 1. Mutants isolated from RIIb strains in the presence of anti-RIIb sera

This experiment was performed with one Inaba-originated and one Ogawa-originated RIIb strain. Two types of mutants isolated from these strains were recognized.

(i) The first type, isolated from RIIb-6973, consisted of a mutant with similar characteristics to those of the smooth agglutinable Inaba strain, indicating the possible reversibility of these non-agglutinable RIIb strains back to original smooth agglutinable forms. Bulging of this mutant appeared within 48 h of incubation. It was shown that its cultural, biochemical and serological characteristics were similar to those of smooth Inaba strain, except that the type exhibited some degree of serological roughness (Table VII).

(ii) The second type of mutant, which was isolated from Ogawa-originated RIIb strain (RIIb-1418) after 72 h of incubation, was a rough form whose cultural, biochemical and serological characteristics were similar to those of a typical rough form of V. cholerae with smooth appearing colonies.

3. 2. Mutants isolated from RIIb strains in the presence of anti (RIIb+S) sera

The same RIIb strains, which were studied in section 3. 1. , were used in this experiment. Three types of mutants were isolated from them.

RIIb-1418 (Ogawa-originated) in serum plates containing anti (RIIb+S) sera; the culture grew after 3 days in a radiated form without definite bulging. The cultural and serological examination of the isolate showed that it was a mixture of two mutants.

(i) The first one was a typical rough (RI) strain with similar characteristics described before for RI strain.

(ii) The second mutant was found out later to be an extremely unstable strain with peculiar behavior. On solid medium the colonies were small, flat, shiny, transparent, but smooth appearing. In liquid medium it showed semi-rough characteristics with moderate turbidity and deposit. Cells in saline, after boiling, gave a stable but granular suspension. This strain was agglutinable with cholera polyvalent and RIIb sera, but not with Inaba or Ogawa factor sera. It was also partially agglutinable with anti-RI serum. While this mutant still contained its type specific antigen, its agglutinability with cholera polyvalent serum but not Inaba or Ogawa factor sera might have been due to the presence of group antigenic factor A of *V. cholerae* but neither B nor C factor. This strain was extremely unstable, and within a few days was converted to a rough form (Table VII).

(iii) In serum plates seeded with Inaba-originated RIIb (RIIb-6973) strain, bulging appeared within 72 h. This new isolate was shown to be an intermediate strain having serological characteristics between RIIa (RIIa with Inaba characteristics) and RIIb strains. On solid medium, colonies were smooth type, raised, semi-transparent and easily suspended. In liquid medium, it gave moderate uniform turbidity, and some deposit, but no surface pellicle. No detectable biochemical changes were observed compared with those of the parent strain. This mutant was agglutinable with polyvalent and Inaba factor sera, as well as anti-RIIa serum, but not as strong as regular smooth Inaba strain. It was also agglutinable with anti-RIIb serum but not with Ogawa factor nor anti-RI sera (Table VII).

3. 3. Mutants isolated from RIIb strains in the presence of anti(RIIb+RI) sera

By this treatment, the first mutant was isolated from RIIb 6973 strain. The mutant, which bulged within 48 h, had the cultural and biochemical properties of a smooth cholera vibrio strain. The uniform cell suspension was agglutinable with polyvalent, anti-RIIa and Inaba factor sera, but not Ogawa factor or anti-RIIb serum. It was only weakly agglutinable with anti-RI. So, serologically it was similar to an Inaba strain.

A second mutant, which bulged within 72 h, was isolated from serum plates containing RIIb-1418 strains. It was an intermediate strain between Inaba-originated RIIa and RIIb, similar to one described in section 3. 2. iii. It was moderately agglutinable with cholera polyvalent, Inaba factor and anti-RIIa, as well as with anti-RIIb sera. But it was neither agglutinable with Ogawa factor nor with anti-RI sera (Table VII).

3. 4. Mutants isolated from RIIb strains in the presence of anti (RIIb+RI+S) sera

RIIb-6973 with this treatment did not produce any bulging, but it grew in a progressive ring type form. Material from the thin layer of the outer ring was selected after 72 h. The isolate strain had a cultural characteristic of a semi-rough form. It was also an intermediate strain between RIIa and RIIb, agglutinable with cholera polyvalent, Inaba factor, and anti-RIIa, as well as with RIIb sera, but neither with Ogawa factor nor with anti-rough sera.

RIIb-1418, under the same conditions, produced radiated type growth as described before. The material of the extremities of the branches was controlled

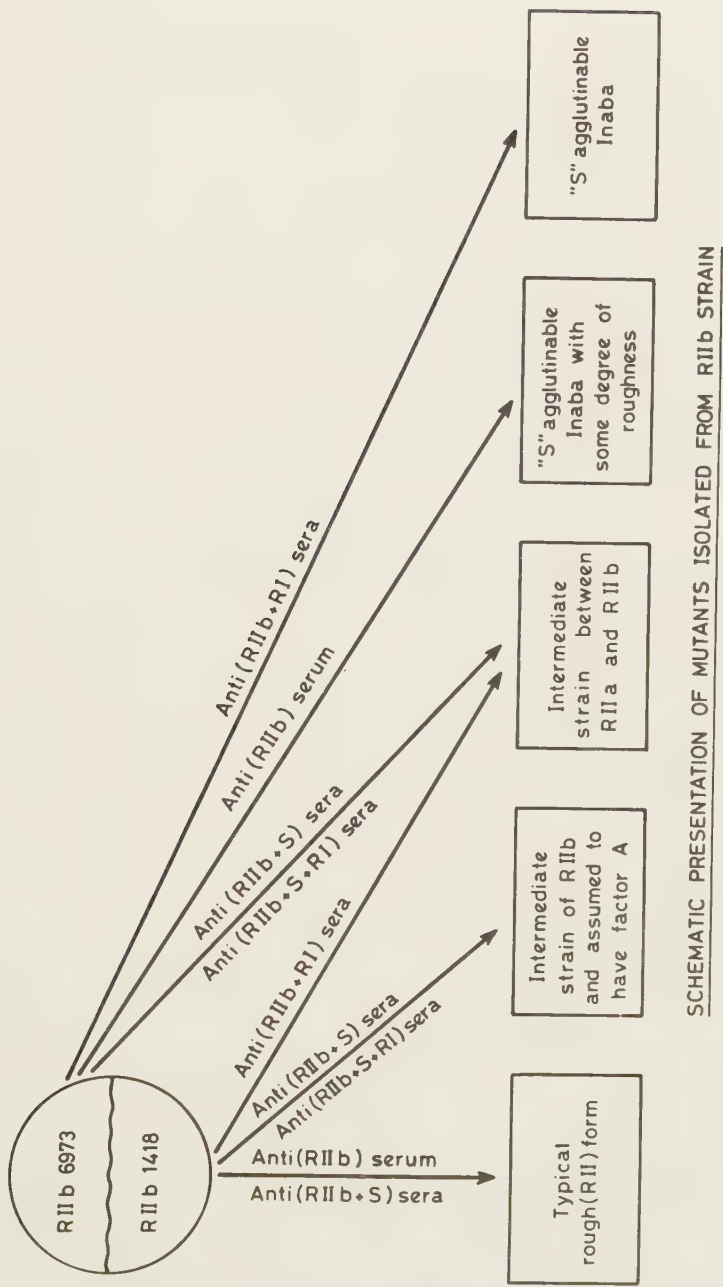


Figure 3

Table VII

Serological behavior of mutants isolated from RIIB strains

Strains Antisera						
	RIIB parent strain	S agglutinable Inaba	S agglutinable Inaba with some degree of roughness	Intermediate strain between RIIBa and RIIBb	Intermediate strain of RIIBb with assumed factor A	(RI)rough form
Anti-RIIB serum	+++ 640	- 0	- 0	++ 160	++ 160	- 0
Cholera polyvalent	w 40	+++ 320	+++ 320	++ 80	++ 80	- 0
Inaba factor serum	w 40	+++ 320	+++ 320	++ 80	- 0	- 0
Ogawa factor serum	- 0	- 0	- 0	- 0	- 0	- 0
Anti-RI serum (cholera X)	w 40	w 40	+ 80	- 0	+ 80	++ 160

Slide agglutination results are graded from the most complete(++++) to the least partial(w),- indicates a negative result. The figures give the titres of the tube agglutination.

after 72 h. The isolate was very unstable, having cultural, biochemical and serological behavior similar to the mutant described under section 3. 2. ii. It was an intermediate strain, agglutinable with cholera polyvalent and RIIB sera, but not with anti-RIIA, Inaba or Ogawa factor sera; it was partially agglutinable with anti-RI serum. This strain was also unstable and changed to a rough form within a few days.

4. Mutants of certain NAG vibrios

In the following experiments for isolation of different mutants, we studied our six NAG vibrios. The cultural, biochemical and serological characteristics of these vibrios have been given in Tables I, II and VI. The schematic presentation of mutants isolated from NAG In-28, NAG Og-14 and NAG N-1, and their serological properties are respectively shown in Figures 4, 5 and 6, and Tables VIII, IX and X.

4. 1. Mutants isolated from smooth strains in the presence of homologous S serum

Rough (RI) mutants were isolated from S parent strains in the presence of corresponding homologous antisera. Bulging of mutant appeared within 48 to 72 h. The isolated mutants had the cultural characteristics of a rough form, similar to those of V. cholerae. No change in biochemical behavior was observed, comparing to that of parent strains.

- RI NAG-28 was agglutinable with its homologous serum at a titre of 1 : 320; with cholera X, and other anti-RI of V. cholerae sera, the mutant was moderate to weakly agglutinable.

- RI NAG-14 gave a titre of 1 : 160 with its homologous antiserum, while weakly agglutinable with cholera X, and other anti-RI of V. cholerae.

- RI NAG-1 was agglutinable with its homologous antiserum at a titre of 1 : 160, but it was weakly agglutinable by cholera X or other RI sera. The serological relationships of these RI strains with other rough forms will be described later.

- RI NAG-23 was partially agglutinated with anti-RI of V. cholerae, with a maximum tube titre of 1 : 80. Its homologous antiserum had a tube titre of 1 : 160.

- The mutant isolated from S NAG A-1 had a semi-rough characteristic. It was not agglutinable with any other anti-S or -RI sera of V. cholerae or other NAG vibrio available, but with its own corresponding anti-S and homologous sera.

- RI NAG B-2 was weakly agglutinable with anti-RI of V. cholerae, and its homologous antiserum had a tube titre of 1 : 320. The serological cross-reactions between RI strains of cholera and NAG vibrios are shown in Table XVII.

In addition to RI form, a second type of mutant bulged out within 48 h in serum plates containing anti-S NAG N-1 cultured with smooth NAG N-1. This type showed colonies of SR type; in liquid medium, it produced uniform turbidity, with a slight amount of deposit. Serologically, it behaved like an intermediate strain, agglutinable moderately with polyvalent and Inaba factor sera, and lightly with S NAG N-1 and RI NAG N-1. Its serological properties are shown in Table X. It should be mentioned that no change in biochemical properties was observed in these SR mutants, compared to those of corresponding parent strain.

4. 2. Mutants isolated from RI strains in the presence of anti-RI serum

When RI strain of NAG In-28 was cultured in serum plates containing corresponding anti-RI serum, a mutant was isolated which appeared to be a complete

non-agglutinable form, with cultural and biochemical characteristics similar to those of smooth parent strain of NAG In-28. Serologically, it behaved like a complete non-agglutinable form of NAG In-28; it was agglutinable with anti-S NAG In-28 serum, and weakly agglutinable with corresponding anti-RI serum, but not with cholera antisera (Table VIII). RI strain of NAG Og-14, in serum plates containing homologous antiserum, produced bulging within 48 h. The isolated mutant had cultural and biochemical behavior of smooth parent strain of NAG Og-14. Serologically, it acted like a complete non-agglutinable form of NAG Og-14, without any detectable agglutination with cholera antisera. It was agglutinable with anti-S NAG Og-14, but not with corresponding anti-RI serum (Table IX). No mutant was isolated from rough forms of NAG N-1, NAG-23, NAG A-1 and NAG B-2 when cultures were made after adjunction of corresponding anti-RI serum.

4. 3. Mutants isolated from S forms in the presence of anti (S+RI) sera

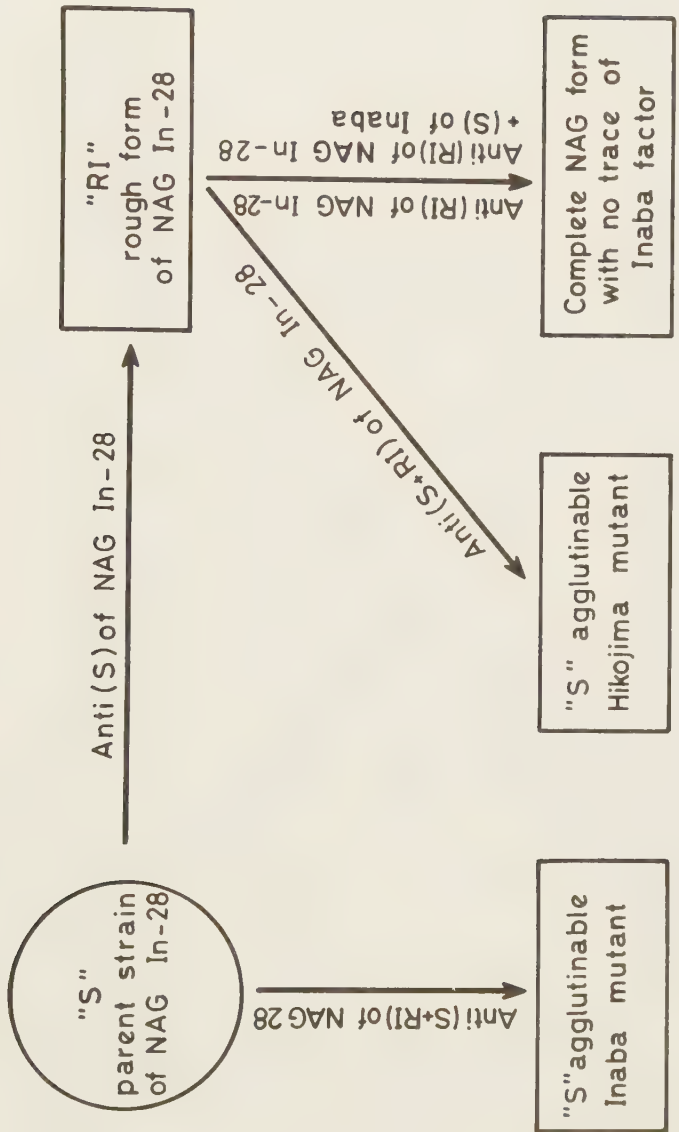
When smooth parent strain of NAG In-28 was cultured in serum plates containing a combination of homologous anti-S and corresponding anti-RI sera, bulging appeared within 48 h. The isolated mutant showed cultural and biochemical characteristics similar to those of *V. cholerae*. Serologically, it behaved like an Inaba strain. It was strongly agglutinated by cholera polyvalent and Inaba factor sera, but not by anti-Ogawa factor, cholera X, corresponding RI or S of NAG In-28 sera. The results of slide and tube agglutinations are shown in Table VIII. Smooth parent strain of NAG Og-14 was cultured in the presence of its homologous antiserum plus corresponding anti-RI serum, in order to isolate a smooth agglutinable form of cholera vibrio. The experiment was performed several times with different concentration of sera, but no mutant was isolated. When smooth parent strain of NAG N-1 was cultured in serum plates containing homologous plus corresponding anti-RI sera, bulging of a mutant appeared in 48 h. This mutant had cultural and biochemical properties of smooth *V. cholerae*. It was an Inaba oriented strain, being moderately agglutinable with polyvalent and Inaba factor serum, but not with Ogawa factor serum. It gave very weak reactions with anti-S and -RI of NAG N-1 (Table X). No mutant was isolated from smooth parent strains of NAG-23, NAG A-1 or NAG B-2 in the presence of their corresponding anti-S plus anti-RI sera.

Smooth parent strain of NAG In-28 and NAG Og-14 were also cultured in the presence of corresponding sera anti-RI plus anti-S of cholera vibrio (anti-Inaba serum). S NAG Og-14 gave rise to a complete NAG Og-14 mutant, agglutinable with anti-S serum of NAG Og-14 only. No mutant was isolated from strain S of NAG In-28 by this treatment.

4. 4. Mutants isolated from RI forms in the presence of anti (S+RI) sera

When RI NAG In-28 was cultured in the presence of corresponding anti (S+RI) sera, a new mutant bulged out in 48 h, which had cultural and biochemical characteristics similar to those of *V. cholerae*. Serologically, this mutant behaved like a Hikojima serotype strain. It was agglutinable with polyvalent, Hikojima, Inaba and Ogawa factor sera. Slide agglutination results with fresh unboiled antigen was stronger than with the boiled one. No agglutination was detected with anti-NAG In-28 and RI sera. The findings of slide tests were confirmed by tube test method using boiled antigen, which are shown in Table VIII.

When RI strain of NAG Og-14 was cultured in serum plates containing cor-



SCHEMATIC PRESENTATION OF MUTANTS ISOLATED FROM NAG In-28

Figure 4

Table VIII
Agglutination pattern of NAG In-28 and its mutants

Strains		"S"	RI	Complete	Inaba	Hiko jima
Antisera	NAG In-28 parent	NAG In-28 mutant	mutant	mutant	mutant	mutant
Cholera poly-valent	+(80)	-(0)	-(0)	-(0)	+++ (320)	+++ (160)
Inaba factor	W(40)	-(0)	-(0)	-(0)	++ (160)	++ (160)
Ogawa factor	-(0)	-(0)	-(0)	-(0)	-(0)	++ (160)
S NAG In-28	++++ (640)	+(40)	+++ (320)	-(20)	-(20)	-(20)
Cholera X	-(0)	W(40)	-(20)	-(20)	-(20)	-(20)
RI NAG In-28	-(0)	+++ (320)	W(40)	W(40)	-(20)	W(40)

Slide agglutination results are graded from the most complete (++++) to the least partial (W) reaction. The figures give the titres of the tube agglutination. 0 indicates a titre less than 1:20.

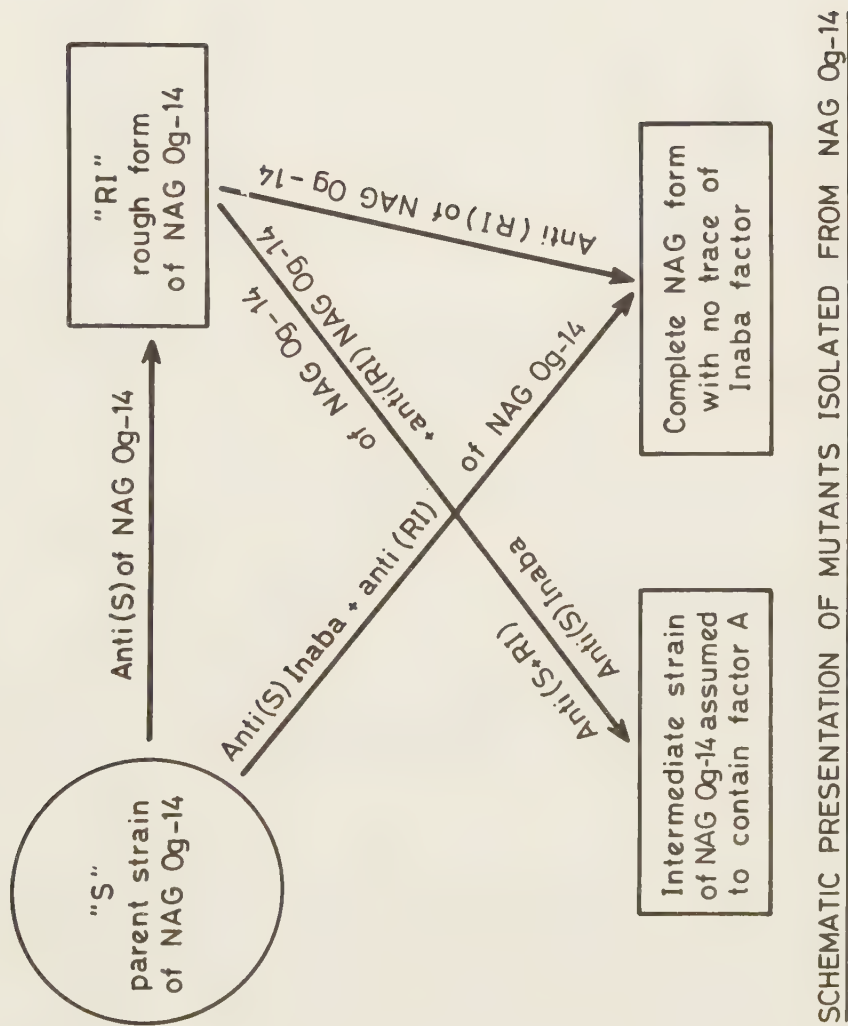
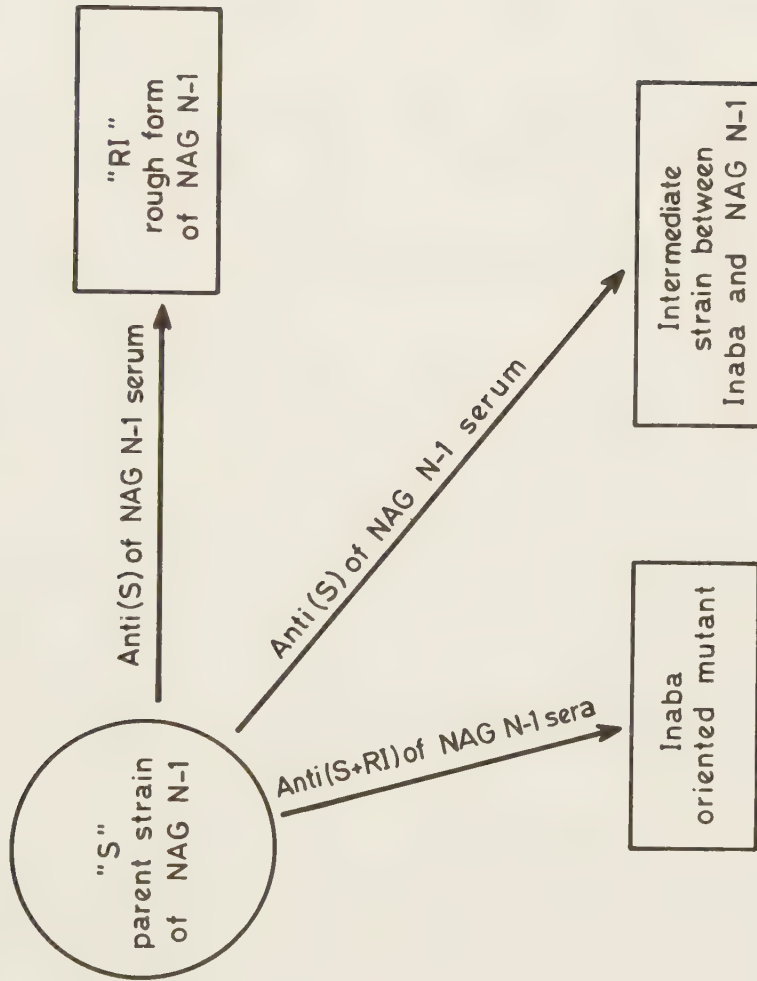


Figure 5

Table IX
Agglutination pattern of NAG Og-14 and its mutants

Strains Antisera	"S" NAG Og-14 parent	RI NAG Og-14 mutant	Complete NAG Og-14 mutant	Intermediate strain of NAG Og-14 assumed to contain fac- tor A
Cholera poly- valent	W(40)	-(0)	-(0)	++(160)
Inaba factor	W(40)	-(0)	-(0)	-(0)
Ogawa factor	-(0)	-(0)	-(0)	-(0)
S NAG Og-14	++++(1280)	W(40)	+++ (320)	++(160)
Cholera X	-(0)	W(40)	-(0)	-(0)
RI NAG Og-14	W(40)	+++ (160)	-(0)	+(40)

Slide agglutination results are graded from the most complete (++++) to the least partial (W) reaction. The figures give the titres of the tube agglutination. 0 indicates a titre less than 1:20.



SCHEMATIC PRESENTATION OF MUTANTS ISOLATED FROM NAG N-1

Figure 6

Table X

Agglutination pattern of NAG N-1 and its mutants
compared with Inaba strain

Strains		"S" NAG N-1 parent	RI NAG N-1 mutant	Intermediate mutant	Inaba oriented mutant	S Inaba <u>V. cholerae</u>
Antisera						
Cholera polyvalent		-(0)	-(0)	++(160)	++(160)	++++(320)
Inaba factor		-(0)	-(0)	++(80)	++(80)	++++(320)
Ogawa factor		-(0)	-(0)	-(0)	-(0)	-(0)
S NAG N-1		++++(640)	-(20)	+(80)	W(20)	-(0)
Cholera X		-(0)	W(20)	-(0)	-(0)	-(0)
RI NAG N-1		-(0)	+++ (160)	+(40)	+(20)	-(0)

Slide agglutination results are graded from the most complete (++++) to the least partial (W) reaction. The figures give the titres of the tube agglutination. 0 indicates a titre less than 1:20.

responding anti (S+RI) sera, a mutant bulged out within 72 h, with a cultural character of a semi-rough form. There was no change in biochemical properties, compared with those of parent strain. Serologically, it behaved like an intermediate strain. It was agglutinable only with polyvalent and anti-S NAG Og-14 sera, but not with Inaba or Ogawa factor sera. It was also weakly agglutinable with anti-RI NAG Og-14 serum. Because of this serological behavior, in addition to the type specific antigenic factor of NAG Og-14, we would conclude that it may contain only group antigenic factor A, which makes it agglutinable with cholera polyvalent serum. This strain was very unstable and changed to a rough form within a few days. No mutant was isolated from RI form of NAG N-1, NAG A-1 and NAG B-2 when cultured in the presence of corresponding anti-S and -RI sera.

RI forms of NAG In-28 and NAG Og-14 were both cultured in the presence of corresponding anti-RI plus anti-S of V. cholerae (Inaba serotype). By this treatment, a complete NAG form, agglutinable with anti-S NAG In-28 only was isolated from RI form of NAG In-28. RI NAG Og-14, in this treatment, gave rise to an intermediate mutant, agglutinable with anti-S of NAG Og-14 and cholera polyvalent sera, but not with Inaba or Ogawa factor sera, similar to the one described before.

B. IMMUNO-SEROLOGICAL INTERRELATIONSHIPS OF SMOOTH, ROUGH AND MUTANTS OF CHOLERA AND NAG VIBRIOS

1. Case of smooth, rough and mutants of cholera vibrio

Cross-agglutination tests were performed by slide and tube methods, using heated antigens against Inaba, Ogawa and anti-rough (cholera X) absorbed factor sera, as well as non-absorbed antisera of smooth, rough and mutants. The results of cross-agglutination between four strains of V. cholerae in the smooth and rough forms are shown in Table XI. As may be expected, smooth strains of Inaba and Ogawa serotypes demonstrated their own specificity, giving only weak cross-reactions with heterologous antisera (titre not more than 1 : 80).

Typical rough (RI) strains lost their O smooth antigenic factors responsible for their type and group specificity, assuming rough serological specificity. Rough forms, because they lack O smooth antigenic determinants, seemed to have a common antigenic factor : all agglutinating titres were close to each other regardless of the sero- or biotypes of their parent strains. These rough forms were often weakly agglutinable with unabsorbed O smooth sera, indicating the existence of anti-rough antibodies in these sera.

Serological cross-reactions between smooth Inaba and Ogawa serotypes and their three mutants are shown in Table XII. It appears that there is a close relationship between smooth Inaba and RIIa strain which is derived from smooth Inaba. This mutant RIIa is agglutinable with cholera polyvalent, and Inaba factor serum only to a titre of 1 : 160 and 1 : 80, respectively. It has been mentioned before that the quantity of RIIa cells required for the absorption of anti-Inaba serum was four times greater than that of Inaba cells. RIIf strain was weakly agglutinable with cholera polyvalent and Inaba factor sera and gave a high titre of agglutination with homologous antiserum.

Table XI

Serological cross-reactions between smooth and rough forms of
V. cholerae

Strains	Inaba 462	Inaba 6973	Ogawa 389	Ogawa 1418	RI 462	RI 6973	RI 389	RI 1418
Antisera								
Cholera polyvalent	+++ 320	+++ 640	++++ 640	++++ 640	- 0	- 0	- 0	- 0
Inaba factor	+++ 320	+++ 320	- 0	- 0	- 0	- 0	- 0	- 0
Inaba 462 *	++++ 320	+++ 160	++ 80	+ 40	W 40	W 40	+ 40	++ 80
Inaba 6973 *	+++ 160	++++ 640	++ 80	++ 80	W 40	- 20	W 40	+ 80
Ogawa factor	- 0	- 0	+++ 320	++++ 320	- 0	- 0	- 0	- 0
Ogawa 389 *	++ 80	++ 80	++++ 640	+++ 320	- 0	+ 80	W 40	++ 80
Ogawa 1418 *	++ 80	++ 80	+++ 320	++++ 320	+ 40	W 20	W 20	+ 40
RI 462 *	- 0	- 0	- 0	- 0	+++ 320	+++ 160	+++ 160	+++ 160
RI 6973 *	- 0	- 0	- 0	- 0	+++ 160	++++ 640	+++ 160	+++ 320
RI 389 *	W 40	- 0	- 0	W 40	+++ 160	+++ 160	++++ 320	+++ 160
RI 1418 *	W 20	- 20	- 0	- 0	+++ 160	+++ 320	+++ 320	++++ 640
Cholera X	- 0	- 0	- 0	- 0	++ 160	++ 160	++ 160	++ 160

* Unabsorbed serum

Slide agglutination results are graded from the most complete(++++)to the least partial(W)reaction. The figures give the titres of the tube agglutination. 0 indicates a titre less than 1:20.

Table XII

Serological cross-reactions between 5 parent strains of V. cholerae and their mutants

Strains Antisera	Smooth Inaba	Smooth Ogawa	Rough form of Inaba/ Ogawa	RIIa mutant of Inaba	RIIb mutant of Inaba/ Ogawa
Cholera polyvalent	++++ 320	++++ 640	- 0	++ 160	W 40
Inaba factor	++++ 320	- 0	- 0	++ 80	W 40
Inaba crude *	++++ 320	++ 80	W 40	++ 80	W 40
Ogawa factor	- 0	+++ 320	- 0	- 0	- 0
Ogawa crude *	++ 80	++++ 640	W 40	+ 80	W 40
Cholera X	- 0	- 0	++ 160	W 40	W 40
RI(Inaba origin) *	- 0	- 0	+++ 320	W 40	W 40
RI(Ogawa origin) *	W 40	W 40	++ 160	W 40	W 40
RIIa crude *	++++ 320	W 20	+ 80	++++ 320	W 80
RIIb crude *	W 40	W 40	W 40	W 80	++++ 320

* Unabsorbed serum

Slide agglutination results are graded from the most complete(++++), to the least partial(W) reaction. The figures give the titres of the tube agglutination. 0 indicates a titre less than 1:20.

The results of cross-absorption of anti-RIIa and RIIb sera are shown in Table XIII. Ogawa factor serum, which contains only anti-B antibody, did not agglutinate RI, RIIa and RIIb mutants; the agglutination of RI, RIIa and RIIb cells by RIIa serum absorbed with Ogawa cells should not be caused by either anti-A or anti-B antibodies; such agglutination could be due either to the anti-C or anti-RI antibodies. On the other hand, the absorption of the same serum (anti-RIIa) with RI cells, absorbed the homologous antibody against RI strain only. Therefore, the agglutination of RI cells with unabsorbed anti-RIIa serum is most likely due to the presence of anti-RI antibody, while agglutination of RIIa and RIIb cells with anti-RIIa serum absorbed with Ogawa cells, is most likely due to the presence of anti-C antibody. Thus, it can be assumed that RIIa serum contained anti-C and -RI antibodies. Ogawa crude serum agglutinated RIIa mutant at a titre of 1 : 80, while Ogawa factor serum did not agglutinate any of the strains : it is probable that those strains are agglutinated by anti-A antibody. So it could be concluded that RIIa serum contains anti-A, -C and -RI antibodies.

As shown in Table XIII, RIIb serum contains a homologous antibody against RIIb cells, which could not be absorbed by either Inaba, Ogawa or RI cells. Therefore, this antibody is distinct from anti-A, -B, -C or -RI antibodies. On the other hand, since RIIb cells are weakly agglutinable with crude Ogawa serum, but not with Ogawa factor serum, this agglutination is not due to antigen B, but rather to antigen A in a small quantity (and maybe RI). Weak agglutinability of RIIb cells with Inaba factor serum indicates the presence of a small quantity of C antigenic factor. Weak agglutination with anti-RI serum is also an indication of the presence of a small quantity of RI antigen. Furthermore, RIIb cells could absorb all antibodies present in anti-RIIa serum, which consisted of anti-A, -C and -RI. Conversely, RIIa cells could absorb antibodies in RIIb serum responsible for agglutination of RIIa and RI cells. Therefore, it can be concluded that anti-RIIb serum contains, in addition to its homologous antibody, antibodies against A, C and RI antigens.

For the confirmation of findings which were described above, absorption ability of different antigens were tested against crude Inaba antiserum (Table XIV). This serum, in unabsorbed state, agglutinated smooth homologous Inaba cells with a titre of 1:320, while the other antigens were agglutinated with a titre of 1 : 80, except RI antigen which gave a titre of 1 : 40. It was previously shown that each antiserum gave the highest agglutinin titre against its own homologous strain (Tables V, VI, XIII). The homologous smooth Inaba cells, as may be expected, caused the total absorption of corresponding antibodies in this Inaba crude antiserum. Absorption with RI cells caused disappearance on only homologous RI antibody responsible for RI cell agglutination. Therefore, crude (unabsorbed) Inaba serum contained antibodies against RI cells.

When the crude Inaba antiserum was absorbed with RIIa cells, using the same quantity of cells as homologous Inaba cells, the absorbed serum did not react with any other antigens, except homologous smooth Inaba cells, but with low titre. This may be due to the quantity of anti-C and/or -A antibodies, which were not totally absorbed by RIIa cells; assumably these antigens are in less quantity or possess a lower combining power in RIIa cells as compared to that in homologous Inaba cells. The absorption of this serum with smooth Ogawa

Table XIII

Cross-absorption of anti-RIIa and -RIIb sera with different antigens

Antisera and condition	Strains	S Inaba (462)	RI (462)	RIIa (462)	RIIb (462)	S NAG In-28	S NAG Og-14
RIIa antiserum(462)	Unabsorbed	320	80	320	80	80	80
	Absorbed with Inaba cells	0	0	0	0	0	0
	Absorbed with Ogawa cells	80 to 160	0	80 to 160	40	40	40
	Absorbed with RI cells	160 to 320	0	160 to 320	40	20	20
	Absorbed with RIIb cells	0	0	0	0	0	0
RIIb antiserum(462)	Unabsorbed	80	40	80	320	80	80
	Absorbed with Inaba cells	0	0	0	320	0	0
	Absorbed with Ogawa cells	40 to 80	0	40	160 to 320	40	40
	Absorbed with RI cells	80	0	40	320	40	40
	Absorbed with RIIa cells	0	0	0	320	0	0

Figures give the titres of tube agglutination, before and after absorption. 0 indicates a titre less than 1:20.

Table XIV

Absorption effect on agglutinability of crude Inaba antiserum							
Strains	S Inaba (462)	S Ogawa (1418)	RI (462)	RIIa (462)	RIIb (462)	NAG In-28	NAG Og-14
Crude Inaba antiserum							
Unabsorbed	320	80	40	80	80	80	80
Absorbed with S Inaba cells(462)	0	0	0	0	0	0	0
Absorbed with S Ogawa cells(1418)	160	0	0	0	0	0	0
Absorbed with RI cells(462)	320	80	0	80	80	80	80
Absorbed with RIIa cells(462)	80 to 160	0	0	0	0	0	0
Absorbed with RIIb cells(462)	160	40	0	0	0	0	0
Absorbed with NAG In-28 cells	80	0	0	0	0	0	0
Absorbed with NAG Og-14 cells	160	40	0	20	0	0	0

Figures are the results of tube agglutination, before and after absorption.
0 indicates a titre less than 1:20.

cells decreased agglutinability of the serum with smooth Inaba cells, which may be due to the absorption of anti-A antibody. But the absorbed serum did not agglutinate other antigens : the C factor, present in Ogawa cells, is either in a small quantity or has low combining power. Absorption with RIIB cells decreased agglutinability of the serum against Inaba and Ogawa cells, while the other antigens were not at all agglutinated with this absorbed serum : the RIIB antigen has absorbed anti-RI, -A and -C. It was observed (Table VIII) that RIIB will be agglutinated by anti-A, -C and -RI antibodies, so that it must be assumed that RIIB cells absorbed A, C and RI antibodies from crude Inaba serum (Table X).

2. Case of smooth and rough forms of certain NAG vibrios

The results of cross-agglutination between six strains of NAG vibrios in the smooth and rough forms are shown in Table XV. We see that each antiserum gave the highest agglutinin titre against its homologous strain. But since these strains had diversified O smooth antigen, they exhibited slight cross-agglutination with each other's antisera if any. Rough forms of our NAG strains lost their O smooth antigenic specificity and exhibited a new serological character. These rough forms, contrary to the RI forms of V. cholerae, were not greatly cross-agglutinable with each other's antiserum. Anti-RI NAG In-28 agglutinated RI NAG Og-14 at a titre of 1 : 80, and anti-RI NAG Og-14 agglutinated RI NAG In-28 at a titre of 1 : 40. The other cross-reactions, if any, were at a titre of 1 : 20 or less. O smooth antisera of these NAG forms did not cross-agglutinate with rough forms at a titre higher than 1 : 40, except the anti-NAG A-1 serum which agglutinated its own rough form to a titre of 1 : 80. Similarly, anti-rough serum of NAG A-1 agglutinated its smooth cells to a titre of 1 : 80, either because smooth strain contained rough antigen, or isolated rough mutant is in fact a semi-rough (SR) form.

3. Case of smooth and rough forms of cholera and certain NAG vibrios

Cross-reactions between four smooth strains of V. cholerae and six smooth strains of NAG vibrios are shown in Table XVI. NAG In-28, and NAG Og-14 were weakly agglutinated by polyvalent and Inaba factor sera, as well as unabsorbed anti-Inaba and Ogawa sera. NAG N-1 was weakly agglutinated with anti-Ogawa 1418 only. Neither of them were agglutinated with Ogawa factor serum. Three other NAG vibrios did not react with any of V. cholerae O antiserum. On the other hand, anti-NAG In-28 and NAG Og-14 sera agglutinated all four V. cholerae strains to a titre of 1 : 80 (exception : Ogawa 1418 with anti-NAG Og-14 serum; the titre was only 1 : 40). Antisera of NAG N-1, NAG-23, NAG A-1 and NAG B-2 did not show any detectable agglutination with V. cholerae strains.

The serological cross-reactions between rough forms of cholera and NAG vibrios are presented in Table XVII. The most significant cross-reaction is between RI NAG In-28 and RI of V. cholerae. All four anti-rough sera of V. cholerae agglutinated RI NAG In-28 strain with a titre of 1 : 80. Similarly anti-RI NAG In-28 gave a uniform titre of 1 : 80 with all four rough strains of V. cholerae. Cross-reactions between rough forms of cholera and NAG Og-14 vibrios were similar to those of NAG In-28, but somehow slightly weaker. Neither rough form of NAG N-1 was agglutinable with anti-RI sera of cholera vibrio, nor did rough forms of cholera vibrio react with anti-RI serum of NAG N-1

Table XV

Serological cross-reactions between smooth and rough forms of NAG vibrios

Strains Antisera	S NAG 28	S NAG 14	S NAG 1	S NAG 23	S NAG A-1	S NAG B-2	RI NAG 28	RI NAG 14	RI NAG 1	RI NAG 23	RI NAG A-1	RI NAG B-2
S NAG-28	+++ 640	+	-	W 40	-	-	W 40	-	-	+	-	W 40
S NAG-14	-	+++ 1280	W 40	-	-	-	+	W 40	W 40	W 40	-	W 40
S NAG-1	-	W 80	+++ 640	-	-	-	-	-	-	W 40	-	-
S NAG-23	-	-	-	+++ 640	-	-	-	-	-	-	-	-
S NAG A-1	-	+	-	-	++ 320	-	-	-	-	-	++ 80	-
S NAG B-2	-	W 40	-	-	-	+++ 320	-	-	-	-	-	W 40
RI NAG-28	-	-	-	-	-	-	+++ 320	+	-	+	-	W 20
RI NAG-14	-	W 40	-	-	-	-	W 40	+++ 160	-	W 20	-	W 20
RI NAG-1	-	-	-	-	-	-	-	-	+++ 160	-	-	-
RI NAG-23	-	-	W 40	-	-	-	-	-	-	+++ 160	-	W 20
RI NAG A-1	-	W 40	-	-	++ 80	-	-	-	-	-	+++ 640	-
RI NAG B-2	-	-	-	-	-	-	-	W 20	-	W 20	-	+++ 320

Slide agglutination results are graded from the most complete(++++)to the least partial(W)reaction.
The figures give the titres of the tube agglutination.

Table XVI

Serological cross-reactions between smooth strains of cholera and NAG vibrios

Strains Antisera	Inaba 462	Inaba 6973	Ogawa 389	Ogawa 1418	NAG 28	NAG 14	NAG 1	NAG 23	NAG A-1	NAG B-2
Cholera polyvalent	+++ 320	+++ 640	++++ 640	++++ 640	+ 80	+ 40	- 20	- 0	- 0	- 0
Inaba factor	+++ 320	+++ 320	- 0	- 0	W 40	+ 40	- 0	- 0	- 0	- 0
Ogawa factor	- 0	- 0	+++ 320	++++ 320	- 0	- 20	- 0	- 0	- 0	- 0
Inaba 462	++++ 320	+++ 160	++ 80	+ 40	W 40	W 40	- 0	- 0	- 0	- 0
Inaba 6973	+++ 160	++++ 640	++ 80	++ 80	+ 40	W 40	- 0	- 0	- 0	- 0
Ogawa 389	++ 80	++ 80	++++ 640	+++ 320	+ 40	W 40	- 0	- 0	- 0	- 0
Ogawa 1418	++ 80	++ 80	+++ 320	++++ 320	++ 80	W 40	W 40	- 0	- 0	- 0
NAG 28	+ 80	+ 80	+ 60	+ 80	++++ 640	++ 80	- 0	W 40	- 0	- 0
NAG 14	W 80	+ 80	W 80	W 40	- 0	++++ 1280	W 40	- 0	- 0	- 0
NAG 1	- 0	- 0	- 0	- 0	- 0	W 80	++++ 640	- 0	- 0	- 0
NAG 23	- 0	- 0	- 0	- 0	- 0	- 0	- 0	++++ 640	- 0	- 0
NAG A-1	- 0	- 0	- 0	- 0	- 0	+ 20	- 0	- 0	++ 320	- 0
NAG B-2	- 0	- 0	- 0	- 0	- 0	W 40	- 0	- 0	- 0	++++ ?

Slide agglutination results are graded from the most complete(+++++)to the least partial (++) reaction.
The figures give the titres of the tube agglutination.

(in a significant titre). As explanation, a weak agglutinability can be assumed since the weak agglutination with cholera X serum indicates the presence of R antigen similar to that of V. cholerae. The observation of some cross-agglutinability between rough forms of V. cholerae and those of certain NAG vibrios can be supported by agglutinin titre shown by cholera RI factor serum (X), which agglutinated rough forms of NAG In-28, NAG Og-14 and NAG N-1 at the titre of 1 : 20 to 1 : 40, while the titre against RI forms of various V. cholerae was 1 : 160, indicating the presence of some antigenic factor similar to that of V. cholerae. The fact that cholera RI factor serum (X) did not agglutinate RI forms of NAG-23, NAG A-1 and NAG B-2 could indicate that those cells do not contain RI antigen similar to that of V. cholerae.

4. Case of mutants of cholera and smooth forms of certain NAG vibrios

The serological reactions of NAG In-28 and NAG Og-14 with various antisera of V. cholerae and its mutants are shown in Table XVIII. NAG In-28 is weakly agglutinable with Inaba, but not Ogawa factor serum, indicating the presence of factor C but excluding factor B of V. cholerae. The mutant is not agglutinated by corresponding anti-RI or anti-RI serum of V. cholerae, which might indicate the absence of R antigen. The agglutinability with crude Ogawa serum, but not with Ogawa factor serum, suggests that the agglutination is due to the reaction of the antigen factor A with its homologous antibody. After absorption with Inaba and Ogawa cells, anti-NAG In-28 serum still agglutinates its homologous cells at a high titre : anti-NAG In-28 serum contains a type specific antibody, corresponding to the type specific antigen present on NAG In-28 cells; this antigen is distinguishable from antigenic factors A, B, C and RI of V. cholerae. Therefore NAG In-28 cells contain a type specific antigen, which constitutes the major antigenic component, plus, at least small quantities of antigen factors A and C.

Anti-NAG In-28 crude serum agglutinated Ogawa cells at a titre of 1 : 80 (Table XIX). As it was shown above, NAG In-28 cells do not contain B antigen and it could be assumed that this agglutination is due to the presence of anti-A. On the other hand anti-NAG In-28 crude serum agglutinated Inaba cells at a titre of 1 : 80, which could be due to anti-A or -C. But when this serum was absorbed with Ogawa cells, the titre against Inaba cells fell to about 1 : 20 to 1 : 40 ! This indicates that, before absorption the agglutination of Inaba cells was due to the anti-A and -C, and after absorption to the anti-C only. Crude anti-NAG In-28 serum agglutinated RI cells with a titre of 1 : 40, which disappeared after absorption with corresponding RI cells because of the presence of small quantities of anti-RI. Therefore anti-NAG In-28 contains, in addition to its type specific antibody, a small quantity of anti-A, C and RI. The discrepancy between the absence of antigen RI on NAG In-28 and the existence of anti-RI in homologous serum is not easy to explain. It could be supposed that in these cells the antigen RI is not well exposed and gives weak combining power, so that a slow agglutination is not expressed in the test.

The agglutination pattern of NAG Og-14 cells (Table XVIII) and the absorption pattern of anti-NAG Og-14 was almost identical with that of NAG In-28, except that anti-NAG Og-14 had its own type specific antibody, which could be absorbed neither with antigens of V. cholerae nor those of NAG In-28. Therefore we can conclude that NAG Og-14, in addition to its type specific antigen

Table XVII

Serological cross-reactions between RI forms of cholera and NAG vibrios

Strains Antisera										
	RI 462	RI 6973	RI 389	RI 1418	RI NAG In-28	RI NAG Og-14	RI NAG N-1	RI NAG-23	RI NAG A-1	RI NAG B-2
RI 462	+++ 320	++ 160	++ 160	++ 160	++ 80	W 40	- 20	+ 40	- 0	W 40
RI 6973	++ 160	++++ 640	++ 160	+++ 320	++ 80	+ 40	- 20	+ 40	- 0	W 40
RI 389	++ 160	++ 160	++ 320	+++ 160	++ 80	+ 40	W 20	+ 40	- 0	+ 40
RI 1418	++ 160	+++ 320	+++ 320	++++ 640	++ 80	+ 40	W 20	++ 80	- 0	+ 80
Cholera X	++ 160	+++ 160	++ 160	++ 160	W 40	+ 40	W 20	- 0	- 0	- 0
RI NAG In-28	++ 80	++ 80	++ 80	++ 80	++++ 320	+ 80	- 0	+ 40	- 0	W 20
RI NAG Og-14	W 40	W 40	++ 80	++ 80	W 40	+++ 160	- 0	W 20	- 0	W 20
RI NAG A-1	- 0	- 0	- 20	W 20	- 0	- 0	+++ 160	- 0	- 0	- 0
RI NAG-23	W 40	W 20	+ 40	+ 40	- 20	- 20	- 0	+++ 160	- 0	W 20
RI NAG A-1	- 0	- 0	W 20	- 0	- 0	- 0	- 0	- 0	+++ 640	- 0
RI NAG B-2	W 40	W 40	W 20	W 40	- 0	W 20	0	W 20	- 0	+++ 320

Slide agglutination results are graded from the most complete(++++)to the least partial(W)reaction.
The figures give the titres of the tube agglutination, 0 indicates a titre less than 1:20.

Table XVIII

Serological reactions of NAG In-28 and NAG Og-14

Antisera \ Strains	Smooth NAG In-28	Smooth NAG Og-14
Cholera polyvalent	+ 80	+ 40
Inaba factor	W 40	+ 40
Inaba unabsorbed	W 40	W 40
Ogawa factor	- 0	- 20
Ogawa unabsorbed	+ 40 to 80	W 40
Cholera X	- 0	- 0
RI(NAG In-28)	- 0	- 0
RI(NAG Og-14)	- 0	W 40
RI(Inaba origin)	- 0	W 20
RIIa(Inaba origin)	W 40	+ 40 to 80
RIIb(Inaba origin)	++ 80	++ 80 to 160

Slide agglutination results are graded from the most complete(++++)to the least partial(W)reaction,- indicates no agglutination. Figures give the tube agglutination titres, 0 indicates a titre less than 1:20.

Table XIX

Absorption effect on agglutinability of anti-NAG In-28 and -NAG Og-14 sera

Antisera	Strains	S NAG In-28	S NAG Og-14	S Inaba (462)	S Ogawa (1418)	RI rough form (462)	RIIa mutant (462)	RIIb mutant (462)
NAG In-28	Crude (unabsorbed)	640	80	80	80	40	40-80	40-80
	Absorbed with NAG In-28 cells	0	0	0	0	0	0	0
	Absorbed with Inaba cells	640	0	0	0	0	0	0
	Absorbed with Ogawa cells	640	40	20-40	0	0	20	20
	Absorbed with RI cells of Inaba	640	40-80	80	80	0	40	40
NAG Og-14	Crude (unabsorbed)	0	1280	80	80	40	40	80
	Absorbed with NAG Og-14 cells	0	0	0	0	0	0	0
	Absorbed with Inaba cells	0	640- 1280	0	0	0	0	0
	Absorbed with Ogawa cells	0	1280	20-40	0	0	20	20
	Absorbed with RI cells of Inaba	0	1280	80	80	0	40	40

The figures give tube agglutination titres before and after absorption of the sera. 0 indicates a titre less than 1:20.

contains also antigen factors A, C and RI in small quantities.

RIIa, RIIf, NAG In-28 and NAG Og-14 strains were agglutinable with crude Inaba serum to a titre of 1 : 80. Absorption of Inaba crude serum with each of these antigens, rendered the serum inactive against three other antigens. Absorption with RI antigen had no effect on agglutinability of this serum against these antigens (Table XIV). Inaba serum contains anti-A, C and RI, and the above named strains, each contains A, C and RI antigens in different quantities; therefore, these cross-agglutinations were due to the A and C and RI antigen-antibody systems. Crude anti-RIIa serum agglutinated NAG In-28 and NAG Og-14 to a titre of 1 : 80. The antibodies responsible for this agglutination could be absorbed with Inaba, but not completely with RI cells (Table XIII). Crude anti-NAG In-28 and NAG Og-14 both agglutinated RIIa cells at a titre of 1 : 40 to 1 : 80. The absorption with Inaba cells rendered these sera inactive against RIIa cells, while absorption with RI cells did not have great effect (Table XIX). This also indicates that anti-RIIa, NAG In-28, NAG Og-14 sera share anti-A, C and most likely RI in small quantity. Similarly, NAG In-28 and NAG Og-14 cells were agglutinated at a titre of 1 : 80 with crude anti-RIIf; they were only partially agglutinated when the serum was absorbed with RI cells, but not at all after absorption with Inaba cells (Table XIII). Meantime crude anti-NAG In-28 and NAG Og-14 sera both agglutinated RIIf cells at a titre of 1 : 40 to 1 : 80. Absorption with RI cells decreased the agglutination titre; absorption with Inaba cells rendered the sera inactive against RIIf cells (Table XIX). This is also an indication of sharing antigen C, A and RI. In conclusion, while NAG In-28, NAG Og-14 and RIIf mutant contain their own type specific antigen, RIIa mutant is a semi-rough mutant of Inaba serotype; all these forms will cross-agglutinate with each other's antisera, due to the sharing of antigen factors A, C and RI of V. cholerae in small quantities.

5. Immunological relationships between smooth and various mutants of cholera and certain NAG vibrios based on precipitation reactions

Immunological relationships between V. cholerae, its mutants and certain NAG vibrios were also demonstrated by immunodiffusion tests. Precipitation reactions between anti-Inaba 6973 crude serum and antigens S, RI, RIIa and RIIf of Inaba 6973 are shown in plate B (Figure 7). Antigen RI (well A) produced a precipitation band with this serum, indicating the presence of anti-RI antibody in Inaba crude serum. The band between serum wells and antigen well RIIa (well B), which has identity with the band of antigen RI (well A) indicates the presence of antigen factor RI in RIIa LPS extract. The fine band near RIIa antigen (well B) is due to antigen factors A and C, because it has identity with the precipitation band of smooth Inaba LPS extract (well C), which contains antigen factors A, C and RI. As we noticed, two bands of RIIa LPS extract have merged into each other, while continuing toward well C. Thus, anti-RIIa 6973 of Inaba origin contains antigenic factors identical to Inaba smooth parent strain. On this slide, there is no detectable precipitation band with antigen RIIf (well D), perhaps due to the weak content of those antigenic factors which are common with smooth Inaba strain.

Plate A (Figure 7) shows precipitation reactions between crude antiserum of Inaba 462 and LPS antigen extracts of homologous, corresponding mutants, NAG In-28 and NAG B-2. Smooth antigen extract (well B) produced a precipitation

band which is due to the factors C and A. Rough antigen extract (well A) and smooth antigen extract (well B) each produced a fine precipitation band which seems to have identity. These bands are most likely due to the reaction between antigen-antibody system of (RI) rough antigenic factor. Antigen extract of RIIb strain has not given any reaction on this slide either, because its common antigen factors with those of smooth Inaba strain are very weak. The major precipitation band of RIIa (well E), which is due to the antigen factors A and C, gave a cross-reaction (partial identity) with that of antigen extract of smooth NAG In-28 (well D). This indicates at least the presence of antigen factors A and C in antigen extract of smooth NAG In-28. The very weak band of NAG In-28 is perhaps due to the small quantity of A and C antigenic factors in this extract. RIIa antigen extract (well E) gave an additional fine band, which could be due to the antigen factor RI, which reacted with antibody against RI antigen separately. There is no detectable precipitation band between antigen extract of NAG B-2 and Inaba serum, which indicates that there is no antigenic relationship between Inaba and this NAG vibrio.

In some cases (plate B), antigenic factor RI, present in smooth Inaba LPS extract, has produced the same precipitation band as that of antigenic factor A and C. Also, in RIIa LPS extract the band due to the antigen factor RI has merged with the band due to the antigen factors A and C. This is because antigenic determinants A, C and RI are the parts of the same antigenic molecules of these LPS extracts. In other cases (plate A, Figure 7), antigenic factor RI, in smooth Inaba, RIIa LPS extracts, has produced separate precipitation bands, probably because antigenic factor RI is present as a separate antigenic molecule as an impurity. However, the presence of factor RI in homologous antigenic molecules of the LPS extract cannot be excluded. In this case, the presence of the impurity could have the following explanation: the mass smooth cell prepared for antigenic extraction may contain RI mutant, although in a small number, its LPS extract could contain enough impurity to produce a separate band with anti-RI antibody present in Inaba crude serum.

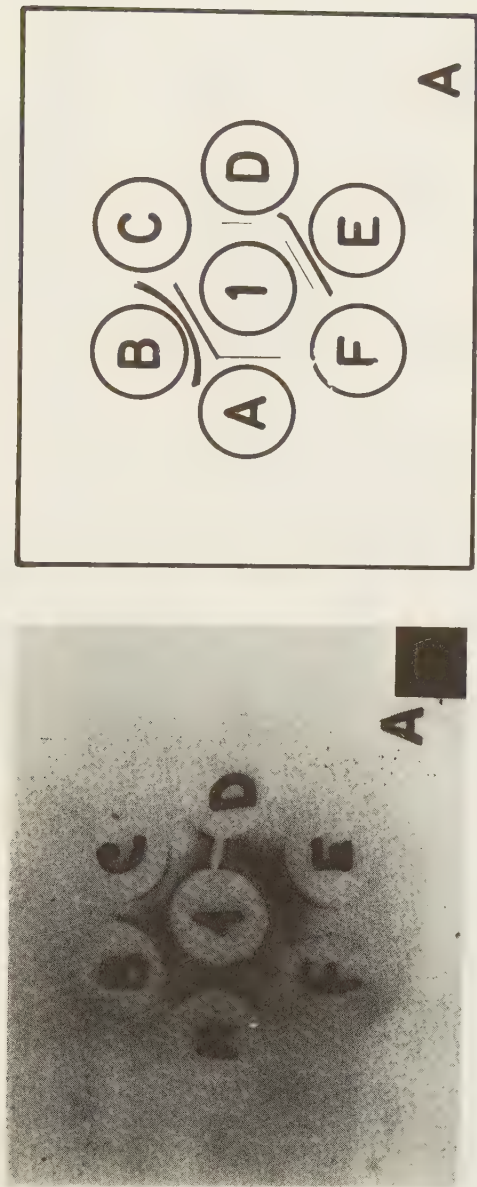
Antigenic relationships between RI, RIIa and RIIb mutants are further shown in plate C (Figure 7). Antigen extract of RI strain (well A) gives two precipitation bands, one with homologous antiserum (well 1), the other with anti-IIb (well 2). These two bands are identical, indicating the presence of anti-RI antibody in RIIb antiserum. These continuous bands join a fine band, which appears between antigen extract RIIb (well B) and anti-RIIb (well 2), indicating the presence of antigenic factor RI in RIIb LPS extract. The heavy band between antigen RIIb (well B) and its homologous antiserum (well 2) represents the antigenic molecule of RIIb, containing its own specific factor plus factors A, C and RI, precipitated by corresponding antibodies in anti-RIIb serum. The band produced between antigen RIIb (well B) and anti-RIIa serum (well 3), shows identity between well C and well 3, as well as between well B and 2. The precipitation band between well C and 3 is due to the homologous antigen-antibody system of RIIa, which contains A and C antigenic factors; therefore, the same identical band between well B and 3 is due to the presence of antigenic factors A and C in antigen extract of RIIb; and the identity of this band with the heavy band between well B and 2 indicates the presence of antigenic factors A and C in antigen molecule of RIIb LPS extract. The separate fine band between well C and 1 is

most likely due to the RI antigenic impurity in RIIa LPS extract. Thus, from these observations, it can be concluded that antigenic LPS extract of RI strain contains only the antigen factor RI, and that anti-RI serum contains only antibody against this factor. RIIa antigenic LPS extract has an antigenic molecule containing factors A, C and maybe RI. Also, in this extract, RI factor is present in a separate molecule, as an impurity. Anti-IIa serum contains antibodies against factor A, C and RI. RIIb antigen extract contains antigen factors RIIb (specific to RIIb strain), A, C and most likely RI; anti-IIb serum contains antibodies against these antigen factors.

In plate D (Figure 7) antigen extract of NAG In-28 (well A) with its homologous serum (well 1) has produced a heavy broad band, most likely due to the interaction of specific antigen factor of NAG In-28, plus antigen factors A, C and RI with their corresponding antibodies. This condensed broad band seems to have partial identity with the band which is produced between antigen NAG In-28 (well A) and anti-IIb serum (well 2), due to the common antigen factors A, C and RI. The latter band also gave a cross-reaction with the band produced between antigen RIIa (well B) and anti-IIb serum (well 2), also due to the antigen factors A and C. The homologous antigen-antibody system of RIIa (well B and 3) produced 3 bands : (i) the one near well 3, which seems merged with the band near well 2, could also be due to the antigen factors A and C; (ii) the one near well B has identity with the fine band between antigen RIIa and serum RIIb (band near well B), which both may be due to antigen factor RI present in the form of an impurity; (iii) the middle band in homologous RIIa system, which is independent; it may be due to the time lag of either antigen or antibody diffusion, but still could represent antigen factors A and/or C.

In plate E (Figure 7) LPS antigen extract of RIIb 462 strain has been set against several antisera. The presence of common antigens A, C and RI ought to show some precipitation band; but in RIIb these antigens are present in a very small quantity, so that the detection of precipitation bands (just like in plates A and B) has not been possible. However, the homologous antigen-antibody system of RIIb produced one heavy band due to the reaction of the specific antigen factor RIIb with its homologous antibody. We observed further a fine band, which could be attributed to the antigen RI impurity.

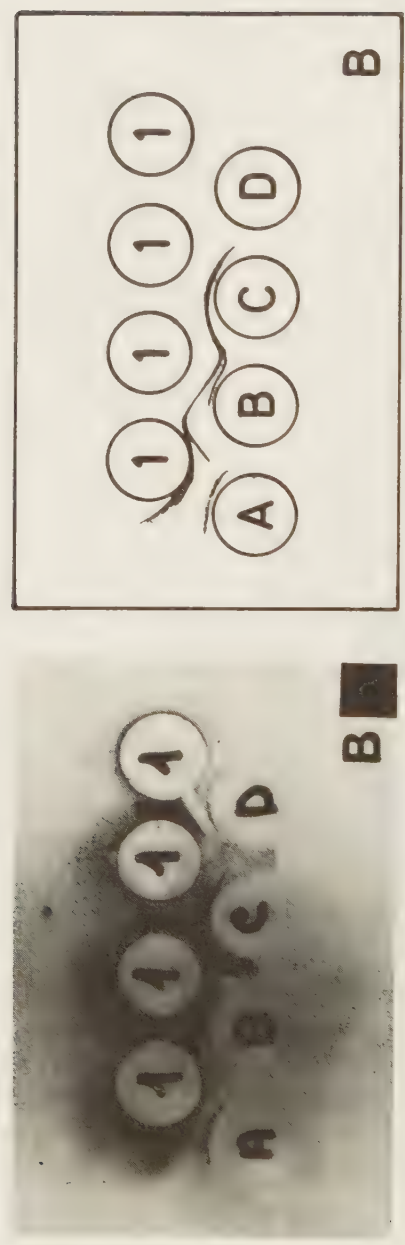
The observations on immunodiffusion tests allow us also to conclude that rough (RI) mutant of *V. cholerae* possesses its own antigenic identity, which is independent from that of smooth form. RIIa mutant, derived from smooth Inaba, contains the antigenic factors A, C and RI. RIIb mutant has its own type specific factor (RIIb) plus small quantities of antigenic factors A, C and RI. NAG In-28, in addition to its own type specific antigen, also contains small quantities of antigen factors A, C and RI. NAG B-2 does not have any antigenic similarity detectable by precipitation reaction with any of the above-named strains.



1=Crude Inaba 6973 anti serum

- | | | |
|-------------|----------|---------------------------|
| A = antigen | RI 462 | D = antigen "S" NAG In-28 |
| B = antigen | S 462 | E = antigen RIIa 462 |
| C = antigen | RIIb 462 | F = antigen "S" NAG B-2 |

Figure 7A



1 = Crude Inaba 6973 anti serum

A = antigen R I 6973

B = antigen RIIa 6973

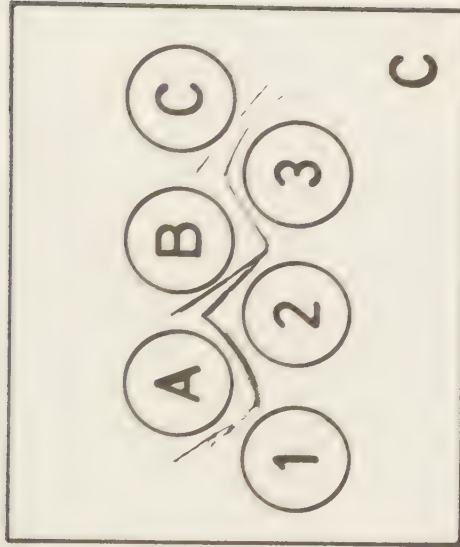
C = antigen S 6973

D = antigen RIIb 6973

Figure 7B

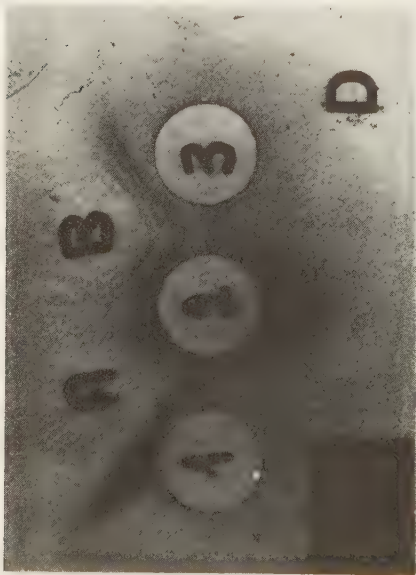


A = antigen RI 462
 B = antigen RIb 462
 C = antigen RIla 462

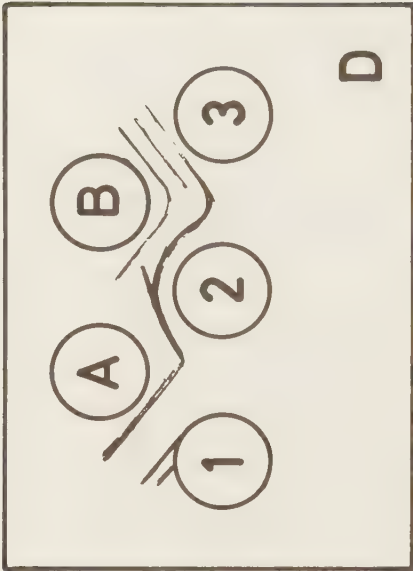


1 = anti RI 462 serum
 2 = anti RIb 462 serum
 3 = anti RIla 462 serum

Figure 7C



A = antigen NAG In-28
B = antigen RIIa 462



1= anti NAG In-28 serum
2= anti RIIb 462 serum
3= anti RIIa 462 serum

Figure 7D



A = antigen RIIb (462)

SERUM:

1 = Cholera polyvalent

2 = "S" Inaba 462

3 = RI Inaba 462

4 = RIIa Inaba 462

5 = RIIb Inaba 462

6 = NAG In - 28 ("S" of)

Figure 7E

IV. DISCUSSION

In recent years, because of their significance in cholera problems, NAG (non-agglutinable) vibrios have received more attention. These problems are important from epidemiological point of view or linked with the pathogenicity and the role of these bacteria as etiological agents in certain cholera-like diarrhea (40). Their bacteriological specification and their laboratory identification are not without interest. It is worthy to mention that, in addition to natural waters and water supplies, NAG vibrios have also been isolated from patients at the end of epidemics, from carriers previously excreting cholera vibrio, and from victims of certain diarrheal outbreaks (1, 67, 77, 95).

In the past, some investigators (13, 86, 94) have hypothesized that NAG vibrio may be the mutant forms of V. cholerae, which has lost its agglutinability in natural water or any other unfavorable environment. Furthermore, it has been claimed that when these NAG vibrios are introduced into the human intestine, they could regain their original O antigenic structure and become agglutinable forms. For the support of this hypothesis, attempts have been made to convert cholera vibrio to non-agglutinable form, and vice-versa, and as we have described in the introduction, several reports on this matter have been presented (11, 12, 29, 94). Until now, this postulation has neither been completely rejected nor accepted. However it must be mentioned that the replacement of somatic antigen of cholera vibrios with that of non-cholera vibrios was reported by genetic hybridization of both organisms (8, 9, 10).

The role of some NAG vibrios as an etiological agent in cholera-like infections, which are increasingly appearing in the form of outbreaks and limited epidemics, has been established (1, 17, 68). Furthermore, it has been shown that some of these NAG vibrios produce an exotoxin, which biologically and immunologically closely resembles that of cholera vibrio (16, 46, 67, 108, 109, 110).

The taxonomy of the vibrios has been studied by several investigators (20, 21, 25, 80, 87). For identification of NAG vibrios, the terms NCV (non-cholera vibrio) or NAG (non-agglutinable) have been suggested (Principles and Practice of Cholera Control, WHO, Geneva, 1970). These terms are for the organisms, which biochemically conform to those of genus Vibrio, possessing flagellar antigen identical to that of V. cholerae, not agglutinable with O and R antisera of the V. cholerae, even after organisms boiled for 2 h. It has also been suggested (55, 80, 82) that NAG vibrios be placed in a single species, V. cholerae, and differentiated from each other by their serological behavior and subdivided into different serotypes according to their O specific antigens.

The conversion of Ogawa to Inaba and smooth to rough forms, in vitro, in

the presence of homologous antiserum has been demonstrated by several investigators. But apparently Ogawa variant has not been isolated from Inaba in vitro. The relationships of these forms have been suggested to be in the form of an antigenic degradation, such as the Inaba is descended from Ogawa by loss of the somatic antigen factor B, while factor C becomes expressive, and rough from smooth by total loss of smooth somatic antigen (81, 91, 102, 103). The effect of the antiserum was assumed to facilitate the selection of spontaneous mutants in the parent cultures (7). Contrary to the observations in vitro, serotypic conversions in both directions have been observed in vivo. It has been observed that in germ free mice, conversions from Inaba to Ogawa and vice-versa and smooth to rough could regularly take place, and these conversions were correlated by the appearance of agglutinating antibody in serum. Apparently, prior vaccination with purified homologous antigen accelerates the conversion of the infecting strain to the heterologous serotypes. On the contrary, the administration of an immunosuppressive agent to mice caused retardation of serotypic conversions (79).

Using the so-called bulging technique (see Material and Methods), we have shown that rough mutants could without difficulty be isolated from smooth forms of V. cholerae of either Inaba or Ogawa serotype in the presence of homologous, or a combination of homologous and heterologous antisera at a level of 3.2 agglutinin units per ml of media. In the case of Inaba strains, addition of anti-Inaba serum is sufficient to prevent the outgrowth of Inaba cells, and consequently, rough mutants are bulging out. In the case of Ogawa, the addition of homologous serum is not sufficient, because Ogawa form will reveal Inaba, as well as rough form. Therefore, addition of both anti-Ogawa and anti-Inaba sera is needed, in order to inhibit the growth of Ogawa cells, as well as its Inaba mutant, and accelerate the isolation of rough form.

Our observations show that it was not possible to obtain any mutant from smooth form of V. cholerae in the presence of the homologous anti-S serum, except rough form. The combination of homologous anti-S plus corresponding anti-RI sera gave also no mutant. It is likely that smooth forms give rise only to a rough (RI) mutant.

The rough form of V. cholerae of either Inaba or Ogawa revealed smooth form of either Inaba or Hikojima types or RIa and RIb mutants, when it was cultured in plates containing anti-RI or a combination of anti-RI plus -S sera, each at a level of 3.2 agglutinin units per ml. The Inaba form was more frequently isolated than the others. On the other hand, when the level of agglutinin units of both anti-RI and -S sera was increased to 6.4 agglutinin units, only RIb forms bulged out. Such an observation may indicate that smooth Inaba or Hikojima type, as well as RIa and RIb, are derived from RI forms, when anti-RI serum alone is present at the level of 3.2 agglutinin units per ml of media; addition of anti-S serum (in the same quantity of agglutinin units) will not be active enough to inhibit the outgrowth of either Inaba, Hikojima or RIa forms. On the contrary, if anti-S level is increased to 6.4 agglutinin units, those forms containing appreciable amount of antigen A and C (such as Inaba, Hikojima and RIa forms) would be prevented from outgrowth; however, mutants of type RIb, which contain negligible quantity of antigen A and C, can still bulge out.

It has been demonstrated that RIb strains are reversible to either smooth Inaba or rough forms; they can also be converted to other intermediate forms.

which have dual antigenic structure of V. cholerae and RIIB mutant. Since RIIB forms are able to mutate in both directions - rough and smooth forms of V. cholerae - the presence of anti-RIIB alone will inhibit only the outgrowth of parent strain, and isolation of both smooth Inaba and rough form would be indiscriminately possible.

The combination of anti-RIIB and -RI sera will inhibit the outgrowth of RIIB parent strain, as well as its rough (RI) mutant. Consequently, smooth Inaba mutant will be driven out. On the contrary, the combination of anti-RIIB and -S sera will inhibit the outgrowth of RIIB parent strain, as well as its smooth Inaba mutant, and as a result, rough mutant will be bulged out.

As may be expected, the combination of anti-RIIB, -S and -RI sera inhibits the outgrowth of RIIB parent strain, as well as its smooth and rough mutants. In serum plates containing either combinations of anti (RIIB+RI), anti (RIIB+S) or anti (RIIB+RI+S) sera, an intermediate mutant was isolated, which has serological properties of RIIB parent strain, as well as the serological properties of RIIa (weak agglutinable Inaba) form. Furthermore, from plates containing anti (RIIB+S) or anti (RIIB+RI+S) sera, another intermediate mutant was isolated, which was agglutinable with anti-RIIB serum, as well as only cholera polyvalent serum (assumably due to the presence of antigen factor A). These observations may indicate that anti-RIIB and anti-S sera are not inhibitory to mutants possessing weak or incomplete antigenic combination of both forms. Thus, such a mutant can grow out from inoculated site of the RIIB parent strain. The relative positions of cholera vibrio mutants to each other and to their parent smooth strain are schematically shown in Figure 8.

Smooth O specific antigens of V. cholerae are composed of a group-specific antigen, factor A, and in addition Inaba and Ogawa serotypes each contain respectively type-specific factors C and B. Hikojima serotype contains the combination of all three factors A, B and C. Rough (RI) forms are the mutants of smooth forms which have lost their smooth O specific antigens, and assumed a new antigenic specificity, due to the exposure of the core structure. The rough antigen is common among all strains of V. cholerae, regardless of their smooth serotype (15, 42, 43, 60, 81, 82, 93, 96).

Inaba-originated RIIa cells agglutinate with crude Ogawa serum, but not with Ogawa factor serum. This indicates that the agglutination is not due to the presence of antigen factor B, but most likely to the factor A and/or RI. Meanwhile, their agglutination with Inaba factor serum must be due to the presence of antigen C. On the other hand, it has been demonstrated that anti-RIIa serum contains antibodies against antigen factors A, C and RI. Therefore, we can conclude that RIIa mutant, derived from the rough form of Inaba strain, contains antigens A, C and RI. Similarly, weak agglutination of RIIB mutant with crude Ogawa (but not Ogawa factor serum) excludes the presence of antigen factor B, and indicates that this agglutination is most likely due to the presence of factor A and/or RI. Their weak agglutinability with Inaba factor serum must be due to the presence of antigen C in small quantity. On the other hand, it has been demonstrated that anti-RIIB serum contains a small amount of antibodies against antigen factors A, C and RI. In addition, the cells of RIIB strain contain type specific antigen RIIB and it has been shown that anti-RIIB serum contains type specific antibody not absorbable with V. cholerae antigen factors A, B, C

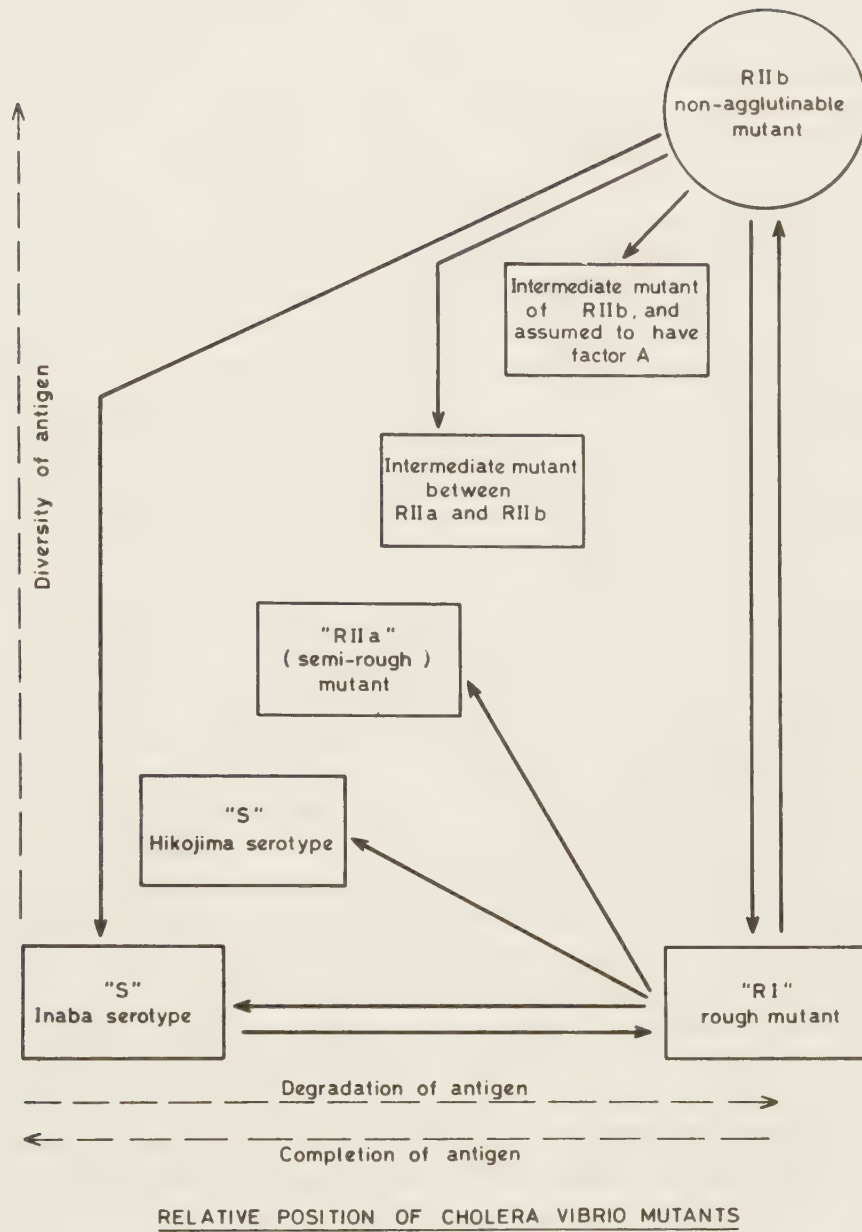


Figure 8

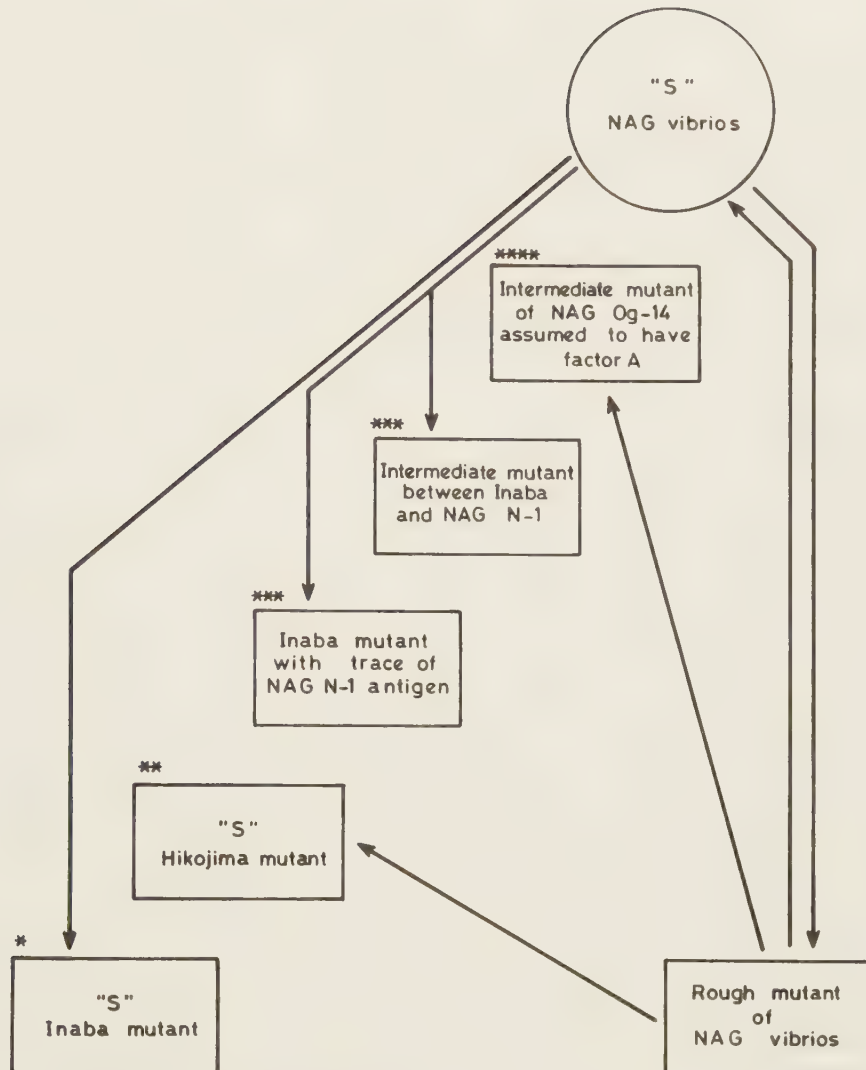
or RI. Therefore, we can assume that, in addition to their own type specific antigen (RIIb antigen), RIIb cells contain small quantities of A, C and RI antigenic factors.

When RI, RIIa and RIIb mutants derived from V. cholerae were checked by routine slide agglutination test and results indicated that RIIa forms may be diagnosed as V. cholerae: these forms were agglutinated with both cholera polyvalent and corresponding factor sera, depending on the serotype of parent strain from which RIIa was derived. The RI form, derived from either Inaba or Ogawa strains, was not agglutinated with cholera polyvalent, Inaba or Ogawa factor sera, but with homologous RI factor serum. In this case, there was no appreciable difference in agglutinability of RI form, regardless of their smooth parent forms; it shows that without using anti-RI factor serum, this mutant may be mis-diagnosed as a non-agglutinable vibrio. The RIIb forms were agglutinated with polyvalent and Inaba factor sera so weakly that the reaction could not be considered as a positive slide agglutination. Anti-RI factor serum also did not show positive agglutination (Table XX). Thus by routine slide agglutination test of V. cholerae, this mutant cannot be diagnosed, either as smooth or as rough form of V. cholerae, but only as so-called NAG. As mentioned already, this RIIb mutant can be derived from rough form of V. cholerae, and revert to the original smooth form. Therefore, we must conclude that non-agglutinable vibrio, based on routine serological diagnosis, does not exclude RIIb mutants of V. cholerae. Factor serum of RIIb form can be prepared by absorption of the homologous serum with Inaba cells; the inclusion of such a factor serum in routine serological diagnosis of cholera is recommended in order to detect this mutant of V. cholerae, which otherwise will be diagnosed as a NAG vibrio.

It has been demonstrated that certain NAG vibrios could be converted to either smooth V. cholerae (Inaba or Hikojima), rough form, or intermediate forms. The rough mutant has been isolated from smooth NAG In-28 in the presence of its homologous serum. If combination of both homologous anti-S and corresponding anti-RI sera are added to the medium, the outgrowth of smooth parent strain as well as its rough mutant is inhibited. This gives a chance to V. cholerae mutant to outgrow (under such conditions an Inaba mutant was isolated). On the other hand, in the presence of homologous anti-RI serum or a combination of homologous anti-RI and anti-S of Inaba sera, rough form of NAG In-28 (which is mutable to either smooth V. cholerae or back to smooth NAG parent strain) will revert to a complete non-agglutinable form of parent strain. When combination of homologous anti-RI and corresponding anti-S sera are added, neither rough form nor smooth parent strain of NAG In-28 will outgrow, but V. cholerae mutant will have the chance to bulge out (in this case, Hikojima serotype has been isolated).

Mutants isolated from either NAG Og-14 or NAG N-1 are not the same as those derived from NAG In-28. However, it seems correct to assume that direction of mutation is basically the same; the effect of each corresponding anti-S or -RI serum, individually or in combination, on S parent strain or rough mutant follows the same principle as for NAG In-28. The relative positions of NAG vibrio mutants to each other and to their parent strain are schematically shown in Figure 9.

We have shown that NAG In-28 and NAG Og-14 contain, in addition to their



- * Obtained from "S" NAG In-28
- ** Obtained from rough NAG In-28
- *** Obtained from "S" NAG N-1
- **** Obtained from rough NAG Og-14

RELATIVE POSITION OF NAG VIBRIO MUTANT

Figure 9

Table XX

Slide agglutination results of smooth cholera vibrio and its mutants

Strains Antisera	Smooth		Rough(RI)		RIIa mutant of		RIIb mutant of	
	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa
Cholera polyvalent	++++	++++	-	-	++	++	W	W
Inaba factor	++++	-	-	-	+	-	W	W
Ogawa factor	-	++++	-	-	-	+	-	-
Cholera X (Anti-rough)	-	-	++	++	-	-	-	-
RIIb (Inaba origin)	W	W	W	W	W	W	++++	++++
RIIb (Ogawa origin)	W	W	W	W	W	W	+++	++++

Slide agglutination results are graded from the most complete (++++)
to the least partial (W) reaction. - indicates no detectable agglutina-
tion.

type specific antigens, small quantities of A and C antigenic factors of V. cholerae; each strain could exist in a form deprived of this common antigenic property; as for NAG N-1, it did not share any antigens with cholera vibrio in the beginning. The Inaba mutant isolated from NAG In-28 is rather strongly agglutinable with cholera polyvalent and Inaba factor serum; the mutant isolated from NAG N-1 is rather weakly agglutinable with those sera, and it could therefore be considered similar to RIIa (semi-rough) mutant of V. cholerae. The intermediate mutant isolated from NAG In-28 possesses the dual serological properties of Inaba and Ogawa, which places it in Hikojima category, the intermediate form of Inaba and Ogawa of V. cholerae. Being agglutinable with cholera polyvalent, Inaba factor and anti-NAG N-1 sera, the intermediate mutant isolated from NAG N-1 has also the dual serological properties of Inaba serotype and NAG N-1. The intermediate mutant isolated from NAG Og-14 is agglutinable with anti-NAG Og-14 and cholera polyvalent sera only, but not with Inaba and Ogawa factor sera; this observation suggests the possession of NAG Og-14 specific antigen, and only of factor A of V. cholerae. Under such conditions, and considering the intermediate mutants isolated from RIIb form (Table VII), we would like to conclude that intermediate forms which contain V. cholerae group (A) or type specific antigen (C), plus other diversified antigen always exist; but due to their instability they are not often recovered.

The observation obtained in the present studies may throw a light on the appearance of non-agglutinable vibrio during inter-epidemic period of cholera. In the course of cholera epidemic, the antibodies against smooth cholera vibrio will increase in individuals of the community due to either mass vaccination or infection (in apparent or inapparent form). These antibodies against smooth form, in vivo, may suppress the growth of homologous smooth strain and produce conditions which favor the overgrowth of rough mutant. This may be the reason why rough form of V. cholerae has often been isolated in cholera convalescents or in the final phase of a cholera epidemic. If individuals produce anti-rough antibody, then further mutation of RI could take place to either smooth form of V. cholerae or RIIb mutant, which may be diagnosed as NAG by the routine sero-bacteriological techniques. It is reasonable to assume that the individuals in a community where a cholera epidemic has occurred have residual immunity against smooth V. cholerae. It is plausible, therefore, that rough form mutates to RIIb form rather than smooth form of V. cholerae, which may pass to the environment. During the inter-epidemic period, antibody level against cholera vibrio in individuals will gradually decrease, while antibodies against non-agglutinable mutants of V. cholerae will increase, due to continuous passage of these mutants from environment to man, or from man to man, with or without clinical symptoms. When in individuals the immunity level against this non-agglutinable mutant of V. cholerae is high enough, a reversion to the original agglutinable form may take place in vivo. The newly converted cholera vibrio may cause an outbreak of a new epidemic in endemic areas. The observation by Sack and Miller (1969) with regard to sero-conversion of Ogawa to Inaba and to rough form, and vice-versa in germ-free mice, may have a correlation to our findings in vitro; these authors noted a close correlation between sero-conversion and increase of agglutinating antibody titre against the inoculated strain, which may support the hypothesis formulated above. However, the au-

thors did not specify the rise of anti-rough antibody, when rough form was inoculated in mice; furthermore, they did not describe the detection of non-agglutinable forms, which may also have appeared, but they have stated that, apparently, the immune response exerts a forceful selective action on the growth of the heterologous serotype vibrios.

The immunochemistry of V. cholerae has been studied by several investigators (62, 63, 69). It has been reported by Jackson and Redmond (1971) that glucose, heptose, mannose, glucosamine, glycerol and ethanolamine are present in lipopolysaccharides extracted from cells of V. cholerae. But 2-keto-3-deoxyoctonate (KDO), which is a component of the core region of most gram negative bacteria, was absent from V. cholerae, and the authors postulated that the cholera vibrio may have a fundamentally different lipopolysaccharide structure from that of Salmonella. In gram negative bacteria, the O specific antigenic side chain is usually composed of a few sugars in the form of repeating units (24, 38, 71, 72, 73). In Salmonella and Shigella, O antigens which contain the same sugars are placed in the same chemotype. The antigens of the same chemotype may carry quite different serological specificities, in other words, they may belong to different serogroups (47, 53, 57, 58, 59, 70, 92); the basic sugar constituents of each chemotype are the same, but within the chemotype, the composition and configuration of repeating units, which provide the specificity for the given antigen, may differ. Any modification to the composition or configuration such as changes in the linkage (1-4 vs 1-6) position between sugars, alteration in omeric configurations (α vs β linkage), attachment of additional monosaccharides at different points on the repeating sequence, deletion or substitution for one of the monosaccharides, presence or absence of acetyl groups on one type of sugar residue, may count for the variation in antigenic specificity. These kinds of modifications are responsible for the existence of more than sixty known O antigens of Salmonella (65). It is not unlikely that such changes in composition and configuration in antigenic determinants of V. cholerae could also account for the large antigenic diversity which is encountered not only in RIIb mutants of cholera, but also in certain NAG vibrios.

Future efforts could be directed toward answering some questions in immunochemistry of cholera vibrio antigens. Some of these questions which constitute the future prospects are : what is the composition of repeating units of somatic antigen of V. cholerae ? What are the antigenic factors A, B and C ? Do they have entirely different chemical composition, or do they just differ from each other by configurational changes ? What constitutes their immunodeterminants ? Which enzymes are responsible for maintenance or changes in structure which account for serological specificity, and what are the defects in enzyme system, if any, of the rough and semi-rough forms in correlation to their antigenic structure ? What accounts for antigenic variation in forms such as RIIb mutant (and some NAG vibrios) ? Is the composition of the specific antigen of this variant entirely different from antigenic factors A, B and C of V. cholerae, or just due to simple changes in configuration causing such a change in serological specificity ?

Another point which should be considered is the mechanism by which these antigenic conversions may take place. We have already, in our studies, considered mutation as a means for these conversions, but we do not have sufficient

evidence to show whether or not this is a spontaneous or induced mutation: does the antiserum simply have an inhibitory effect, or does it induce the mutation ? For Bahaskaran and Gorrill (1957), the serotypic conversion of Ogawa to Inaba, and that of smooth to rough form, are due to spontaneous mutants already present in culture; antiserum would simply facilitate their isolation. The authors have also stated that for the conversion from Ogawa to Inaba the number of cells inoculated must be 10^7 or more and that conversion from S to R was detectable even when the size of inoculum was 10^3 cells. However, it is difficult to accept that such a rate (10^{-3}) would be due to the spontaneous mutation.

Other possibility of sero-conversion was described by Bhaskaran and Sinha (1971, 1971a, b), who demonstrated hybridization between V. cholerae and certain non-cholera vibrio, in which the chromosomal region coding for antigenic specificity was transferred from a non-cholera vibrio to cholera vibrio. They have demonstrated that conjugation in cholera vibrio is mediated by a sex factor, designated P factor. Another transmissible factor, designated V factor, has been suggested by the same authors to be present in a non-cholera vibrio strain, which will interact with P factor. Both of these factors have been regarded as plasmids.

It has been shown (65, 78, 92; Simmons, 1971) that changes in antigenic structure and specificity in Salmonella and Shigella could be mediated by bacteriophages. As an example we can mention the lysogenic conversion in group E of Salmonella, in which the presence of phages will alter the composition or configuration of oligosaccharide units of the specific side chain and transform the serological specificity. The possibility of a lysogenic conversion should also be considered in the case of antigenic variation in vibrios.

V. CONCLUSIONS

1. Vibrio cholerae can mutate to various forms in the following manner :

- a) It can mutate to rough form.
- b) The rough form can either reverse back to smooth V. cholerae or mutate to a non-agglutinable (RIIb) form.
- c) The RIIb form can either reverse back to rough form or mutate to smooth V. cholerae. This non-agglutinable mutant, which has never been described in the past, possesses a new serological identity distinguishable from those of V. cholerae and its other mutants. Therefore, in order to detect this new mutant, it is important that anti-RIIb factor serum be included in routine serological diagnosis of V. cholerae.
- d) Other various intermediate mutants, which have partial antigenic structure of V. cholerae (RIIa semi-rough mutant), or dual antigenic structure of V. cholerae and RIIb mutant also occur.

2. Certain so-called NAG vibrios, but not all, can mutate to V. cholerae (serotype Inaba or Hikojima) in the following manner :

- a) Smooth NAG vibrios can either mutate to their own rough form, or smooth form of V. cholerae.
- b) The rough form can either reverse back to smooth NAG form or mutate to V. cholerae.
- c) Other intermediate mutants, which have partial but dual antigenic structure of the corresponding parent NAG strain and V. cholerae, also occur.
- d) The NAG vibrios in the smooth state, each possesses its own serological identity distinguishable from each other's, and those of cholera vibrio. They may or may not contain trace amounts of V. cholerae antigens.
- e) In the rough form, certain NAG vibrios have serological relation to rough form of V. cholerae.

VI. RESUME

Les buts qui ont suscité notre étude furent les suivants :

- (i) l'isolement et la caractérisation de différents mutants de Vibrio cholerae;
- (ii) la réversion de ces mutants à la forme lisse originale et agglutinable de V. cholerae;
- (iii) la conversion de certains vibrions NAG (non agglutinables) en formes agglutinables de V. cholerae;
- (iv) la clarification des relations immuno-sérologiques entre les vibrions cholériques lisses (S) agglutinables et leurs différents mutants et
- (v) la démonstration d'une corrélation sérologique entre des mutants de vibrions cholériques et certains vibrions non agglutinables.

Pour isoler des mutants, nous avons préparé des sérums anti-0 que nous avons mis en présence des souches parentales correspondantes; les sérums étaient incorporés à un milieu semi-solide. En l'absence d'antisérum, une souche mobile pousse en s'étalant progressivement : le lendemain, la surface entière de la plaque est recouverte. La présence de l'antisérum 0 homologue provoque une aggrégation des cellules; elle exerce un effet inhibiteur sélectif et empêche le rayonnement de l'inoculum. Si, dans la population bactérienne ensemencée, il existe un mutant dont l'antigène somatique n'est pas affecté par l'antisérum, l'étalement n'est pas empêché; il y a "bourgeoisement" du mutant : le bourgeon constitué par le mutant se développe en débordant les limites de la population bactérienne ("colonie") et peut être ainsi isolé facilement.

Il a été démontré que les formes S de V. cholerae donnaient des mutants rugueux (R). Les formes R peuvent (i) soit retourner à la forme S de V. cholerae, (ii) soit donner des mutants non agglutinables (dénommés RIIB dans notre étude). Par la suite, la forme RIIB peut réverser à la forme R ou retrouver la forme S de V. cholerae. Nous avons encore isolé d'autres formes de mutants, mutants intermédiaires, qui avaient conservé une partie de la structure antigénique de V. cholerae semi-R (dénommés RIIA dans notre étude) ou qui possédaient une structure antigénique double associant celle de V. cholerae à celle du mutant RIIB.

Certains vibrions NAG sont capables (i) soit de donner des formes R, (ii) soit de muter vers la forme S de V. cholerae (sérotypes Inaba ou Hikojima). Quant à la phase R de ces vibrions NAG, elle peut soit réverser vers la forme S NAG, soit glisser vers la forme S de V. cholerae. Ces mutants agglutinables dérivant de vibrions NAG particuliers ne peuvent pas être différenciés, ni biologiquement, ni sérologiquement des sérotypes Inaba ou Hikojima de V. cholerae. Nous avons, en outre, isolé des mutants intermédiaires dont la structure antigénique correspondait à la fois à celle de la souche parentale NAG et à celle de V. cholerae.

L'analyse antigénique des différents mutants a permis de démontrer que le mutant RIIA de V. cholerae est une forme en phase S-R qui contient des facteurs

antigéniques identiques à ceux de la souche parentale S, mais en moindre concentration. Bien que renfermant des traces d'antigène Inaba, le mutant RIIb possède une antigénicité qui lui est propre; il se différencie sérologiquement des formes S et R de V. cholerae.

Dans leur phase S, les vibrions NAG montrent une antigénicité qui est particulière pour chacun et qui permet de les différencier soit entre eux, soit de V. cholerae. Ils sont capables ou non de renfermer des traces de facteurs antigéniques appartenant au vibron cholérique. Les formes R de certains vibrions NAG possèdent une fraction antigénique R commune avec la forme R de V. cholerae.

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